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Research article

Regulation of glutamine synthetase 1 and amino acids transport in the phloem of young wheat plants

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ABSTRACT

The possible regulation of amino acid remobilization via the phloem in wheat (*Triticum aestivum* L.) by the primary enzyme in nitrogen (N) assimilation and re-assimilation, glutamine synthetase (GS, E.C. 6.3.1.2) was studied using two conditions known to alter N phloem transport, N deficiency and cytokinins. The plants were grown for 15 days in controlled conditions with optimum N supply and then N was depleted from and/or 6-benzylaminopurine was added to the nutrient solution. Both treatments generated an induction of GS1, monitored at the level of gene expression, protein accumulation and enzyme activity, and a decrease in the exudation of amino acids to the phloem, obtained with EDTA technique, which correlated negatively. GS inhibition by metionine sulfoximide (MSX) produced an increase of amino acids exudation and the inhibitor successfully reversed the effect of N deficiency and cytokinin addition over phloem exudation. Our results point to an important physiological role for GS1 in the modulation of amino acids export levels in wheat plants.

1. Introduction

In wheat plants, nitrogen (N) absorbed from soil is assimilated mainly in mature leaf mesophyll cells and used for leaf protein synthesis or exported as amino acids to sink tissues via the phloem [12]. During organ senescence proteins are hydrolyzed to amino acids that are also loaded to the phloem and translocated to new developing tissues [11], such as young leaves and grains. This remobilization process is an important factor in the final grain protein concentration in cereals and legumes [1]. However, the way in which amino acid export to the phloem is regulated is far from clear.

Glutamine synthetase (GS, E.C. 6.3.1.2) is a key enzyme in the assimilation of inorganic N into organic forms. GS catalyzes the ATP-dependent condensation of ammonium with glutamate (Glu) to yield glutamine (Gln), which then provides N groups, either directly or via Glu for the biosynthesis of virtually all the other organic N-containing molecules [16]. There are a number of GS isoenzymes, classified according to their subcellular localization as cytosolic glutamine synthetase (GS1) and chloroplastic/plastidic glutamine synthetase (GS2), which are assumed to play non-overlapping roles [10].

Genetic studies revealed that generally GS2 is encoded by one gene per haploid genome [2,26]. The major role of this isoenzyme is to assimilate ammonium resulting from the reduction of nitrate and to re-assimilate the one released during photorespiration [25]. On the contrary, several genes have been identified encoding GS1 (i.e., four in Arabidopsis [21], three in rice [20] and five in maize [8]), which is predominantly expressed in roots. In shoots its activity level varies between tissues and between the species examined [29]. The function of GS1 is not yet fully elucidated, but several studies suggest different roles for each different gene, which are differentially expressed among organs by internal and external signals [17,28]. A phloem-specific GS isoenzyme has been identified in C3 plant species, and its physiological role appears to be related either to N storage under stress conditions [4] or to N export during plant growth and development [32,33]. In senescing leaves of tobacco, *Gln1-3* is specifically induced in the cytosol [5], concomitantly with chloroplastic protein degradation, suggesting the involvement of this isoenzyme in the re-assimilation of ammonium released during this catabolic process [34]. While, Martín et al. [28] demonstrated the functional importance of cytosolic GS (*Gln1-3 and Gln1-4*) in the control of yield and its components in maize.

N stress is one of the main causes of leaf senescence in field-grown wheat plants, promoting proteolysis and N remobilization

Abbreviations: BAP, 6-benzylaminopurine; EDTA, ethylene-diamine-tetraacetic acid; Gln, glutamine; Glu, glutamate; GMH, γ-glutamylhydroxamate; GS, glutamine synthetase; GS1, cytosolic glutamine synthetase; GS2, plastidic glutamine synthetase; MSX, metionine sulfoximide; N, nitrogen.

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and, ultimately, leaf senescence. Furthermore, the rate of amino acid export from the leaf to the phloem is highly dependent on N plant availability [7]. On the other hand, cytokinins are a class of plant hormones known to delay the senescence process. During the development of senescence induced by N deficiency, the sharp fall in cytokinin concentration is the first event observed before protein degradation [9]. In addition, transgenic tobacco over-expressing cytokinins specifically in senescent leaves, accumulated more N in the older leaves than in the younger ones [22].

In order to improve our understanding of the regulation of N assimilation and recycling in wheat, we studied the activity, protein abundance and gene expression of the isoforms of GS in relation with phloem transport of amino acids after N starvation and 6-benzylaminopurine (BAP, a synthetic cytokinin) application.

2. Results

2.1. Effect of N starvation and BAP addition

Forty-eight hours after N starvation was imposed to 15-day-old wheat plants (minus N plants), there was a decrease in amino acid but not in sugar concentration in the phloem exudates (Fig. 1A). In the last fully expanded leaf (Fig. 1B) there was a slight decrease in amino acid and protein content, while sugar concentration remained constant. The western blot analysis of GS1 and GS2 proteins in leaves (Fig. 2A) indicated a slight decrease in the accumulation of the 44 kDa band corresponding to GS2 subunit and a 26% increase in GS1 subunit (40 kDa) (Fig. 2B). The separation of GS1 and GS2 activities through anion exchange chromatography (Fig. 2C) showed a 40% increment in GS1 activity and a fall in GS2

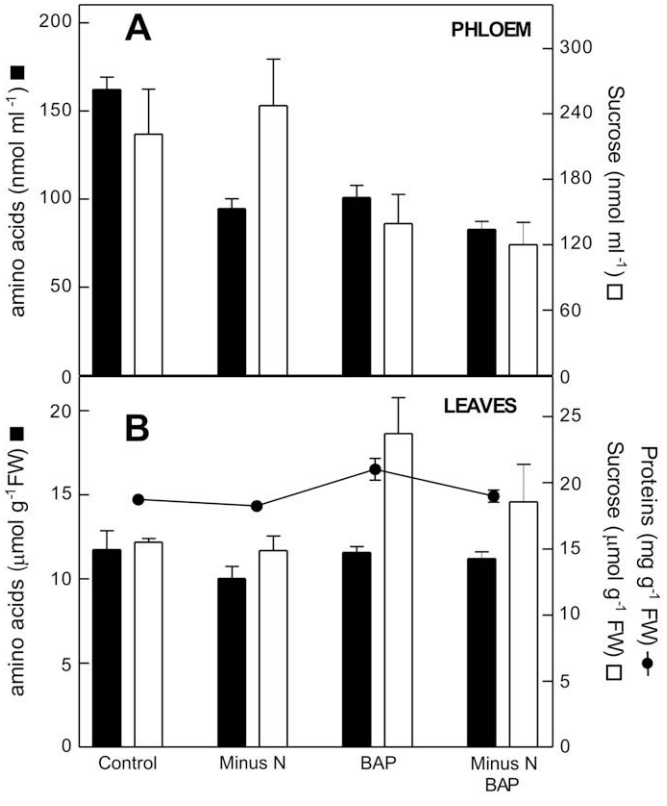

Fig. 1. Effect of N starvation and BAP addition: Amino acid and sugar contents in phloem exudates (A) and amino acid, sugar and protein concentrations in leaves (B) of control plants, N-starved plants (minus N), BAP-supplied plants (BAP) and N-starved and BAP-supplied plants (minus N/BAP). Each value is the mean ± SE of five replicates.

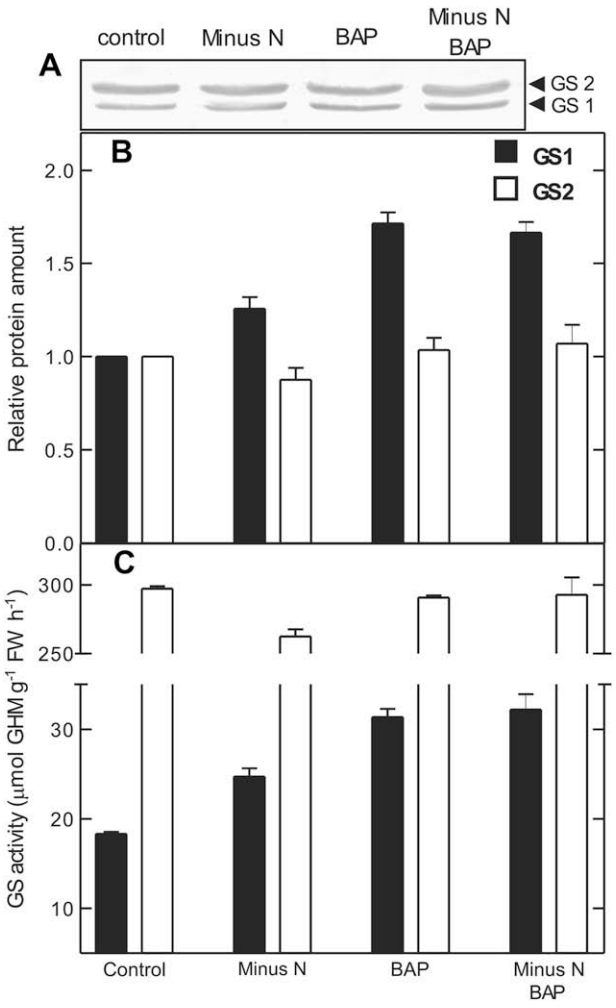

Fig. 2. Effect of N starvation and BAP addition: Western blot (A), band quantification (B) and GS1 and GS2 activity expressed as micromoles of γ-glutamylhydroxamate (GHM) per g FW per tour, measured after separation in an anion exchange chromatography (C) of plants submitted to the treatments detailed in Fig. 1. Each value is the mean ± SE of three replicates.

activity in the same proportion as that observed in the protein amount (12%). Total GS activity decreased (data not shown), due to the low contribution of GS1 in respect of GS2 in leaves (6%).

In hexaploid wheat, there are ten different mRNA sequences published in GenBank coding for GS protein. Therefore, we performed a Neighbor joining phylogenetic tree (Fig. 3) with these sequences and found them to be aggregated in four groups, one corresponding to GS2 transcripts (named *Gs2* group) and three to GS1 transcripts (named *Gs1*, *Gsr* and *Gse* groups). We designed a pair of primers for each group because a high degree of homology was found between the genes inside them (≥96%). The level of expression of *Gs1* (*Gs1a*, *Gs1b* and *Gs1c* transcripts) and *Gs2* (*Gs2a*, *Gs2b* and *Gs2c*) groups was similar while *Gsr* (*Gsr1* and *Gsr2*) was significantly lower and Gse group (*Gse1* and *Gse2*) was not detected at all (Fig. 4A). N starvation induced a 25% increase in *Gs1* expression but a 40% and 12% decrease in *Gsr* and *Gs2* expression, respectively (Fig. 4B).

Plants supplied with 20 μM BAP (BAP plants) presented a decrease in amino acid and sugar contents in the phloem exudates (Fig. 1A). In the leaf tissue, on the contrary, increased protein and sugar but not amino acids concentrations (Fig. 1B). Meanwhile, GS1 protein (Fig. 2B) and activity (Fig. 2C) increased a 70% and *Gs1* and *Gsr* expression increased a 40% and 70%,

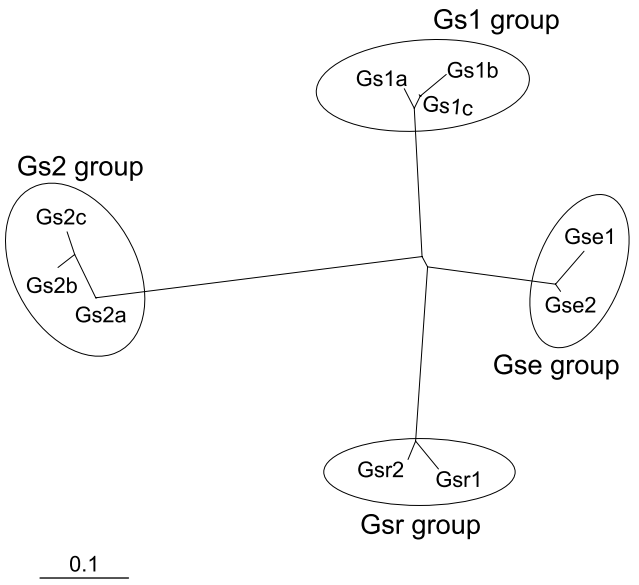

Fig. 3. Unrooted Neighbor joining phylogenetic tree of wheat GS transcripts. Analysis was carried out using BioEdit software [14] and the tree was made with TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Sequences were aggregated into four groups named: *Gs2* group (GenBank accession nos.: *Gs2a*, DQ124212; *Gs2b*, DQ124213 and *Gs2c*, DQ124214), *Gs1* group (GenBank accession nos. *Gs1a*, DQ124209; *Gs1b*, DQ124210 and *Gs1c*, DQ124211), *Gsr* group (GenBank accession nos. *Gsr1*, AY491968 and *Gsr2*, AY491969) and *Gse* group (GenBank accession nos. *Gse1*, AY491970 and *Gse2*, AY491971).

respectively, in response to BAP addition (Fig. 4). Finally, there was no change in GS2 protein abundance (Fig. 2B), activity (Fig. 2C) and transcript level (Fig. 4) with respect to the control plants.

When plants were submitted to N starvation and BAP addition altogether (minus N/BAP plants) protein, amino acid and sugar concentrations remained constant in the leaves (Fig. 1B) while the amino acid and sugar contents decreased in the phloem exudates (Fig. 1A). The level of expression (Fig. 4), protein (Fig. 2B) and activity (Fig. 2C) of GS2 were similar to that of control and BAP plants and higher than minus N plants. Also GS1 activity, polypeptide and *Gs1* expression (Figs. 2 and 4) were similar to BAP plants, while *Gsr* expression was higher than control but lower than BAP plants (Fig. 4).

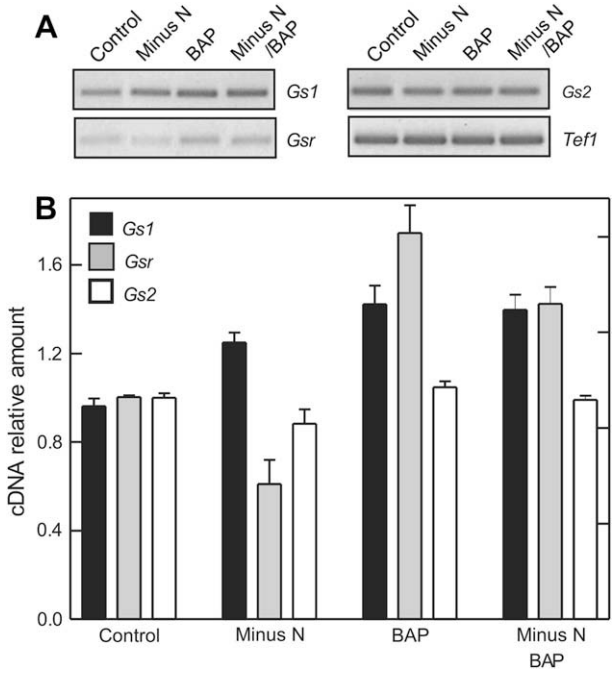

Fig. 4. Effect of N starvation and BAP addition: Expression of *Gs1*, *Gsr* and *Gs2* groups and *Tef1* amplified by RT-PCR for 26 cycles in the case of Tef1, *Gs1* and *Gs2* and 29 cycles for *Gsr* genes (A), and *Gs1*, *Gsr* and *Gs2* band quantifications normalized against Tef1 level of expression (B) of plants submitted to the treatments detailed in Fig. 1. Each value is the mean ± SE of three replicates.

2.2. Effect of MSX

The plants sprayed with different concentrations of the GS irreversible inhibitor, metionine sulfoximide (MSX) presented a gradual decrease of total GS activity reaching a 90% of inhibition at 10 μmol MSX/pot (Fig. 5A), and showing a dose-dependent increase in the ammonium concentration in leaves (Fig. 5B). The concentration of amino acids and sugars in leaves remained constant (Fig. 5C, D), while leaf dry weight/fresh weight ratio (Fig. 5F) slightly increased, presumably due to tissue dehydration and most probably responsible for the observed increment in protein concentration in leaves (Fig. 5E). The amino acid concentration in the phloem exudates increased in proportion to the MSX concentration and GS inhibition, reaching a 50% higher value than the control (Fig. 5G). The sugar concentration in the phloem, though, remained constant at all drug doses (Fig. 5H). Finally, GS protein abundance and transcript levels increased in all MSX doses applied (data not shown).

2.3. Amino acid composition in leaves and phloem exudates

The amino acid composition of leaf tissue (Table 1) and phloem exudates (Table 2) of plants (control, minus N, BAP and MSX) was analysed to better understand the effects of these treatments and the relationship between GS activity and amino acid exudation to the phloem. In leaves, the concentration of Glu decreased in minus N and BAP plants and increased in MSX plants. Inversely, Gln greatly decreased after MSX treatment, but did not fall with N starvation and increased with BAP addition. As a consequence of N starvation other amino acids also decreased (Ala, Asp, Gly, Phe and Thr) but the relative proportion of each amino acid remained unchanged, whereas MSX addition produced an increase in some amino acids (Asn, His, Iso, Leu and Thr) (Table 1).

In phloem exudates Gln droped in all the treatments, but its relative proportion only decreased in the MSX treated-plants. Glu instead, raised in MSX and fall in minus N and BAP plants. Once again, N starvation generated a diminution in the concentration of several amino acids (Ala, Asp, Cys, Gly, Ser and Thr) but dew to the fall in Glu (45%) the proportion of the rest remained constant or even increased. BAP addition decreases the concentration of Arg, Asp, Ser and Thr, but given again the great decline in Glu (60%) only the relative proportion of Arg decreased. Finally MSX caused the increase in Ala, Asp, Gly, Thr and Tyr and a decrease in Cys (Table 2).

2.4. Effect of N starvation and BAP addition combined with MSX

Subsequently we tested whether the repressor effect of N starvation and BAP addition on amino acid phloem exudation would be reversed by the inhibition of GS (Table 3). Total GS activity remained constant in BAP plants, slightly decreased in minus N plants and fell to a 30% in the plants supplied with 5 μmol MSX/pot alone (MSX plants) or in combination with N starvation (minus N/MSX plants) or BAP addition (BAP/MSX plants). Ammonium concentration increased in minus N, MSX, BAP/MSX and minus N/

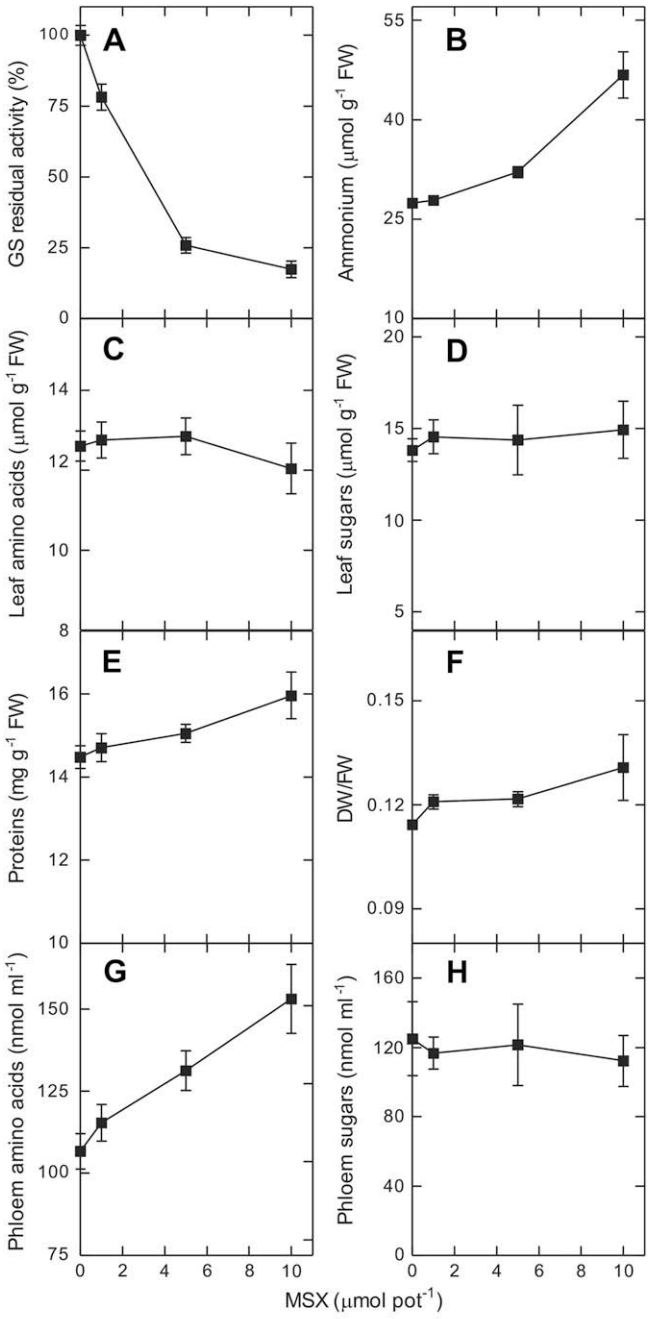

Fig. 5. Effect of different MSX doses: GS residual activity (A), ammonium (B), amino acid (C), sugar (D) and protein (E) concentrations in leaves, leaf dry weight/fresh weight (DW/FW) ratio (F) and amino acid (G) and sugar (H) contents in phloem exudates of plants submitted to different doses of MSX. Each value is the mean ± SE of five replicates.

MSX plants, being higher in the latter due to an additive effect of both treatments. Protein concentration did not decrease in minus N/MSX plants as in minus N plants, and increased in BAP/MSX plants at the same extend as in BAP plants. Amino acid concentration in leaves decreased only in minus N plants, with or without MSX. Finally, addition of MSX to minus N (minus N/MSX) or BAP (BAP/MSX) plants reversed the inhibition in the amino acid export to the phloem which returned in minus N plants to the same level of control plants, and in the BAP/MSX plants raised to a concentration similar to MSX plants (Table 3).

3. Discussion

This study was performed to analyze the involvement of GS enzyme in the regulation of the amino acid export rate established under different external conditions imposed to young plants growing in steady state, and to provide new insights into the physiological role of the different GS1 isoenzymes of wheat.

Many previous reports supported the hypothesis that GS is involved in the control of N status and remobilization in higher plants, including wheat (see [18]). The role played by the different GS isoenzymes is species specific and strongly dependent on both the organ examined and its developmental stage. In wheat, it was observed that during stem and leaf development, the tissue localization of the different forms of GS evolves during the source-to-sink transition in order to ensure optimal utilization of N, particularly during the grain-filling process [23]. However, there are no previous reports on the pattern of expression of the different GS genes in this species.

Here, the pattern of expression of the different genes coding for GS obtained in wheat was similar to its homologous genes in rice [33]. In fully expanded leaves *Gs1* group (homologous to the rice leaf isoform) predominate by far *Gsr* group (homologous to the rice root isoform), while *Gse* is homologous to the isoform specifically expressed in the spikelet, which explains the lack of signal found in our experiments, all performed in leaves. Besides, Ishiyama et al. [20] observed in rice a reciprocal response of the leaf and root isoforms transcripts to ammonium supply, which is coincident with the reciprocal response observed in this work for *Gs1* and *Gsr* groups in minus N plants (Fig. 4).

N deficiency has a repressor effect over *Gs2* expression that BAP efficiently reversed, but BAP alone had no direct effect over the enzyme. *Gsr* expression was also repressed by N deficiency but was highly induced by BAP, regardless of plant N status, although in minus N the induction was significantly lower, likely due to a negative interaction of both stimuli. *Gs1* expression though was induced by N deficiency and BAP addition with no additive effect between them (Figs. 2 and 4). These results denote a differential response of each gene group to the external stimuli applied and show the sensitive modulation of all GS genes by N availability while they suggest a direct cytokinin effect only over GS1 genes (*Gs1* and *Gsr* groups) and that the reversion of N repression of *Gs2* is an indirect consequence of the cell metabolic changes produced by the hormone.

We found a correspondence between activity, protein and gene expression for GS2 isoform. In terms of the level of expression of the different GS1 genes analysed, the 40 kDa band revealed by the western blot might be composed principally by *Gs1* group products. Effectively the induction of *Gs1* expression and GS1 protein were of the same extent (25%) regardless of a 40% repression of *Gsr* genes (Figs. 2 and 4).

In minus N plants the induction of *Gs1* expression and GS1 protein abundance was lower than the enzyme activity, which suggests that N starvation not only triggers *Gs1* genes expression but activates the enzyme as well. There are previous reports of GS1 being subjected to post-translational modifications [27]. In BAP treatment instead, the enzyme activity was comparable with the level of protein abundance and higher than the induction of *Gs1* transcript group expression, which might indicate a diminution in the turnover of this protein. In a recent work we observed that BAP indeed decrease the rate of protein degradation and inhibit the export of assimilates leading to a transformation of source leaves into sink organs (Criado, unpublished results).

Plants N-starved and/or BAP-treated, showed a reciprocal response of the amino acid export rate and GS1, but not GS2, protein amount and activity (Fig. 2). Interestingly, we found

Table 1
Amino acid composition in leaf tissue of control plants, N-starved plants (minus N), BAP-supplied plants (BAP) and MSX treated-plants (MSX). Each value is the mean ± SE of three replicates; ND, not detected; asterisks indicate significant differences between means ($p < 0.05$) of each treatment respect to control plants.

Amino acid	Control		Minus N		BAP		MSX	
	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%
Ala	3.08 ± 0.15	23.4	2.26 ± 0.14	*21.2	3.18 ± 0.11	23.2	3.00 ± 0.36	23.4
Arg	0.13 ± 0.01	1.0	0.12 ± 0.02	1.2	0.11 ± 0.01	0.8	0.12 ± 0.01	0.9
Asn	0.17 ± 0.01	1.3	0.12 ± 0.01	1.2	0.20 ± 0.02	1.5	0.23 ± 0.02	*1.8
Asp	2.50 ± 0.22	19.0	1.91 ± 0.08	*18.0	2.60 ± 0.12	19.0	2.69 ± 0.17	21.0
Cys	0.02 ± 0.01	0.1	0.01 ± 0.01	0.1	0.01 ± 0.01	0.1	0.01 ± 0.01	0.1
Gln	2.53 ± 0.29	19.2	2.39 ± 0.04	22.5	3.49 ± 0.41	*25.5	1.37 ± 0.18	*10.7
Glu	1.16 ± 0.01	8.8	0.84 ± 0.06	*7.9	0.62 ± 0.13	*4.5	1.41 ± 0.04	*11.0
Gly	0.09 ± 0.01	0.7	0.05 ± 0.01	*0.5	0.11 ± 0.01	0.8	0.11 ± 0.01	0.8
His	0.08 ± 0.01	0.6	0.07 ± 0.01	0.7	0.07 ± 0.01	0.5	0.11 ± 0.01	*0.9
Iso	0.22 ± 0.02	1.7	0.22 ± 0.03	2.0	0.24 ± 0.00	1.8	0.31 ± 0.02	*2.4
Leu	0.30 ± 0.02	2.3	0.27 ± 0.04	2.6	0.32 ± 0.01	2.3	0.42 ± 0.02	*3.3
Lys	0.12 ± 0.01	0.9	0.12 ± 0.02	1.2	0.12 ± 0.01	0.9	0.13 ± 0.01	1.0
Met	0.06 ± 0.01	0.5	0.06 ± 0.01	0.6	0.07 ± 0.01	0.5	0.06 ± 0.02	0.5
Phe	0.28 ± 0.02	2.1	0.21 ± 0.02	*2.0	0.23 ± 0.02	1.7	0.28 ± 0.02	2.2
Pro	0.10 ± 0.01	0.8	0.05 ± 0.01	0.5	0.07 ± 0.01	0.5	0.09 ± 0.01	0.7
Ser	1.14 ± 0.13	8.7	0.91 ± 0.01	8.6	0.99 ± 0.15	7.2	1.07 ± 0.07	8.4
Thr	0.64 ± 0.02	4.8	0.50 ± 0.02	*4.7	0.67 ± 0.01	4.9	0.79 ± 0.02	*6.2
Trp	ND		ND		ND		ND	
Tyr	0.17 ± 0.01	1.3	0.15 ± 0.02	1.4	0.18 ± 0.02	1.3	0.19 ± 0.01	1.5
Val	0.40 ± 0.02	3.0	0.33 ± 0.04	3.1	0.41 ± 0.00	3.0	0.41 ± 0.14	3.2
Total	13.18	100	10.63	100	13.68	100	12.80	100

a strong curvilinear relation between the amino acid content in the phloem and the level of expression of *Gs1* group (Fig. 6A). However, we did not observe any clear dependence of the amino acid export to the phloem and the expression level of neither *Gsr* nor *Gs2* groups, since no obvious correlation was found between them (Fig. 6B, C). MSX promoted a notable increase in the export of amino acids but not of sugars to the phloem (Fig. 5E, F). This increment is mainly due to the increase in Glu (Table 2), suggesting a specific accumulation of the former in response to the inhibition of GS enzyme. The herbicide effect of the drug became notable at concentrations above 10 μmol/pot and produced not only a diminution of the amino acid and sugar export but of leaf protein concentration as well (data not shown). The induction of GS genes in response to MSX was also seen in radish and the authors attributed it to a change in the leaf Gln/Glu ratio [36]. Here, we actually observed a two-fall decrease in this ratio after MSX treatment (Table 1). Finally, the treatment of minus N or BAP plants with MSX reversed completely the inhibition of amino acid exudation to the phloem (Table 3).

The rate of amino acid export from the leaf to the phloem is dependent on the N available to the plant and this N is used for synthesis of leaf protein when the supply is low, exported to the phloem when the supply is adequate, and accumulated in the leaf when the supply is above plant demand [7]. The concentration of most amino acids in the phloem exudates is proportional to their leaf concentration. However, some amino acids are either favoured

Table 2
Amino acid composition in phloem exudates of control plants, N-starved plants (minus N), BAP-supplied plants (BAP) and MSX treated-plants (MSX). Each value is the mean ± SE of three replicates; ND, not detected; asterisks indicate significant differences between means ($p < 0.05$) of each treatment respect to control plants.

Amino acid	Control		Minus N		BAP		MSX	
	nmol ml^{-1}	%	nmol ml^{-1}	%	nmol ml^{-1}	%	nmol ml^{-1}	%
Ala	9.87 ± 0.62	5.1	6.31 ± 0.11	*5.3	11.32 ± 0.28	10.1	11.84 ± 0.65	*4.7
Arg	1.65 ± 0.05	0.9	1.36 ± 0.07	1.1	0.23 ± 0.12	*0.2	1.42 ± 0.31	0.6
Asn	ND		ND		ND		ND	
Asp	46.46 ± 0.84	24.1	28.22 ± 0.43	*23.5	27.52 ± 0.64	*24.5	55.18 ± 2.43	*21.9
Cys	1.51 ± 0.19	0.8	0.78 ± 0.09	*0.6	1.13 ± 0.03	1.0	1.04 ± 0.07	*0.4
Gln	18.54 ± 0.59	9.6	12.92 ± 3.24	*10.8	12.19 ± 0.80	*10.9	12.03 ± 0.54	*4.8
Glu	82.36 ± 2.68	42.8	47.97 ± 0.23	*39.9	34.87 ± 1.71	*31.1	131.35 ± 5.00	*52.1
Gly	1.43 ± 0.09	0.7	1.05 ± 0.14	*0.9	1.79 ± 0.01	1.6	1.87 ± 0.03	*0.7
His	ND		ND		ND		ND	
Iso	2.44 ± 0.24	1.3	2.28 ± 0.05	1.9	2.06 ± 0.14	1.8	2.94 ± 0.27	1.2
Leu	2.42 ± 0.31	1.3	2.61 ± 0.08	2.2	2.11 ± 0.22	1.9	3.39 ± 0.45	1.3
Lys	0.87 ± 0.08	0.5	0.99 ± 0.11	0.8	0.58 ± 0.05	0.5	0.85 ± 0.11	0.3
Met	1.25 ± 0.07	0.7	0.99 ± 0.05	0.8	1.00 ± 0.05	0.9	1.12 ± 0.06	0.4
Phe	1.85 ± 0.09	1.0	1.66 ± 0.12	1.4	1.07 ± 0.06	1.0	2.38 ± 0.38	0.9
Pro	ND		ND		ND		ND	
Ser	13.27 ± 0.56	6.9	6.87 ± 0.34	*5.7	9.93 ± 0.42	*8.9	13.94 ± 1.11	*5.5
Thr	6.33 ± 0.18	3.3	3.95 ± 0.09	*3.3	4.97 ± 0.20	*4.4	8.05 ± 0.51	*3.2
Trp	ND		ND		ND		ND	
Tyr	ND		ND		ND		1.58 ± 0.27	*0.6
Val	2.19 ± 0.18	1.1	2.12 ± 0.10	1.8	1.45 ± 0.28	1.3	3.26 ± 0.47	1.3
Total	192.45	100	120.09	100	112.23	100	252.23	100

Table 3
Effect of N starvation and BAP addition in combination with MSX. Glutamine synthetase (GS) activity and ammonium, protein and amino acid concentrations in leaves and amino acid content in phloem exudates of control plants, N-starved plants (minus N), BAP-supplied plants (BAP), MSX treated-plants (MSX), N-starved and MSX supplied plants (minus N/MSX) and BAP and MSX supplied plants (BAP/MSX). Each value is the mean ± SE of five replicates, and the superscript terms "a, b and c" within each column indicate significant differences between means ($p < 0.05$).

Treatments	Leaves				Phloem
	GS (%)	Ammonium μmol g^{-1} FW	Proteins mg g^{-1} FW	Amino acids μmol g^{-1} FW	Amino acids nmol ml^{-1}
Control	100.0 ± 6.7^a	27.1 ± 0.7^a	18.3 ± 0.6^a	15.8 ± 0.2^a	172.5 ± 8.8^a
Minus N	79.9 ± 1.9^b	29.9 ± 0.7^b	16.1 ± 0.9^b	13.0 ± 0.7^b	112.2 ± 13.9^b
BAP	102.2 ± 4.4^a	26.1 ± 1.3^a	21.7 ± 0.9^c	16.1 ± 1.4^a	126.4 ± 18.1^b
MSX	32.5 ± 4.9^c	31.7 ± 1.3^b	19.4 ± 0.7^a	14.8 ± 0.7^a	213.3 ± 7.6^c
Minus N/MSX	29.8 ± 5.9^c	37.7 ± 1.4^c	18.7 ± 0.5^a	12.6 ± 0.2^b	175.1 ± 11.3^a
BAP/MSX	23.6 ± 5.2^c	30.6 ± 1.1^b	22.9 ± 0.7^c	15.7 ± 1.8^a	226.0 ± 16.4^c

or discriminated against their export to the phloem [7] and, as a consequence, the extent of their decrease in the phloem of the plants subjected to N deficiency and BAP addition does not necessarily reflects the changes in its concentration in the leaves (Tables 1 and 2).

Studies performed in rice [33] suggest that during nitrogen remobilization the leaf cytosolic GS1 is important for the synthesis of Gln, the major form of reduced nitrogen in the phloem sap of rice [15]. In wheat, though, Glu is the most abundant amino acid in the phloem of young vegetative plants (Table 2) [7], and Gln only became the predominant one at the final stage of grain filling [31]. As a consequence, the induction of GS1 may decrease the phloem amino acid concentration of young plants, but may increase it during grain filling, by changing the cytosolic Glu to Gln ratio and altering the pool of the preferable amino acid available for export. Indeed, Glu and Gln concentrations changed accordingly to GS1 activity and the exportation of Glu was highly altered with the present treatments (Tables 1 and 2). These observations enable us to suggest a repressor effect of GS1 activity over the phloem amino acid export of young plants and explain the enhanced capacity of transgenic wheat over-expressing GS1, to accumulate N mainly in the grain, reported by Habash et al. [13].

As a conclusion, we suggest that the changes produced in the export of amino acids to the phloem in response to the different physiological conditions here tested might be a consequence of the changes produced in the activity of *Gs1* family products. It still remains to be elucidated if this GS isoform is the major checkpoint controlling the N status of the plant.

4. Methods

4.1. Cultivation

Wheat seeds (*Triticum aestivum* L. cv. pro-INTA, Isla Verde) were sown onto 300 ml plastic pots filled with vermiculite and watered daily with nutrient solution [19] containing 10 mM KNO_3 as the only N source. Plants were grown in a growth cabinet with a constant temperature of 25 °C, a photoperiod of 16 h and a photosynthetic photon flux density of 300 μmol m^{-2} s^{-1} provided by General Electric® 400 W HPL lamps.

4.2. Experiment design

Effect of N starvation and BAP addition: Fifteen-day-old plants grown as described in Section 4.1 were divided into 4 groups and submitted to the following treatments: Control, the same nutrient solution (10 mM KNO_3); minus N, the nutrient solution depleted of KNO_3 (0 mM KNO_3); BAP, the nutrient solution supplemented with BAP (10 mM KNO_3, 20 μM BAP) and minus N/BAP, BAP was added to the nutrient solution depleted of KNO_3 (0 mM KNO_3, 20 μM BAP). All the solutions were replaced after 24 h and 24 h later (48 h of treatment) the youngest fully expanded leaf of each plant was harvested and used immediately for collection of phloem exudates or stored at −80 °C for biochemical and molecular analyses.

Effect of MSX: Fifteen-day-old plants grown as described in Section 4.1 were sprayed 24 h before sampling with the GS irreversible inhibitor metionine sulfoximide (MSX) diluted in 0.08% Tween 20 in different doses (0, 1, 5 or 10 μmol MSX/pot). The youngest fully expanded leaf of each plant was harvested and used immediately for collection of phloem exudates or stored at −80 °C for biochemical and molecular analyses.

Amino acid composition in leaves and phloem exudates: Fifteen-day-old plants grown as described in Section 4.1 were divided into 3 groups and submitted to the following treatments: Control, the same nutrient solution (10 mM KNO_3); minus N, the nutrient solution depleted of KNO_3 (0 mM KNO_3) and BAP, the nutrient solution supplemented with BAP (10 mM KNO_3, 20 μM BAP). All the solutions were replaced after 24 h and a subgroup of the control plants was sprayed with 5 μmol MSX/pot (MSX). Twenty-four hours later (48 h of treatment) the youngest fully expanded leaf of each plant was harvested and used immediately for collection of phloem exudates or stored at −80 °C.

Effect of N starvation and BAP addition in combination with MSX: Fifteen-day-old plants grown as described in Section 4.1 were divided into 3 groups and submitted to the following treatments: Control, the same nutrient solution (10 mM KNO_3); minus N, the nutrient solution depleted of KNO_3 (0 mM KNO_3) and BAP, the nutrient solution supplemented with BAP (10 mM KNO_3, 20 μM BAP). After 24 h all the nutrient solutions were replaced and half of the plants of each group were sprayed with 5 μmol MSX/pot. Twenty-four hours later the youngest fully expanded leaf of each plant were harvested and used immediately for collection of

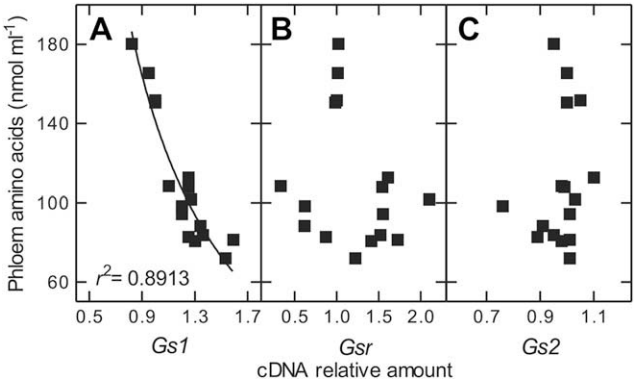

Fig. 6. Scatter plots between amino acid content in phloem exudates and *Gs1* transcript group (A), *Gsr* transcript group (B) and *Gs2* transcript group (C) level of expression. Each point represents one individual sample analysed in the 'Effect of N starvation and BAP addition' experiment.

phloem exudates or stored a −80 °C for biochemical and molecular analyses.

4.3. Collection of phloem exudates

The phloem exudates were obtained as we described before [7]. The youngest fully expanded leaf of each plant was excised and the cut ends of five leaves were pre-incubated in 20 mM ethylene-diamine-tetraacetic acid (EDTA) (pH 8) for 15 min at 25 °C in the dark. Afterwards the leaves were rinsed, transferred to fresh solution, and kept in the same condition for three hours. The exudation solution (1 ml) was stored at −80 °C. For the analysis of amino acid composition the solution was concentrated by freeze-dry.

4.4. Biochemical analysis

The leaves (250 mg fresh weight (FW)) were frozen in liquid N_2 and ground in a mortar. The fine powder obtained was extracted in 1 ml buffer: 50 mM Tris–HCl (pH 7.5); 5 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 10,000 × g for 30 min. The supernatant obtained was used for determination of soluble protein [3], amino acids [37], ammonium (Nessler reagent, Merk) and sugar concentrations [38].

The amino acid composition analysis was performed at the Molecular Structure Facility of UC Davis (http://msf.ucdavis.edu/) with an L-8900 Hitachi amino acid analyzer, which utilized a lithium citrate buffer system followed by a "post-column" ninhydrin reaction detection system. The samples were extracted in water following the procedure describe above and the supernatant obtained was concentrated by freeze-dry, re-suspended in HCl 0.1 N, acidified with sulfosalicyclic acid and freeze overnight prior to injection.

4.5. Immunodetection of GS isoforms

The immunodetection of GS isoforms was carried out by western blot after their separation by SDS-PAGE 15% [24] and transferred to a PVDF membrane (Immobilon, Millipore) [35], using a rabbit antiserum raised against recombinant GS1 of pine [6]. The same amount of extract (3 mg FW) was loaded into each lane.

4.6. Separation of GS isoforms and assay of GS activity

Leaves (4 g FW) were processed as described above and the supernatant was desalted on a Sephadex G-25 column (Amersham Pharmacia) and the isoforms were separated using a Mono Q-Sepharose fast flow (Amersham Pharmacia) equilibrated at pH 7.5 and eluted with a linear gradient from 0 to 550 mM of NaCl. Two activity peaks were obtained, corresponding to GS1 and GS2, eluted at 230 and 400 mM NaCl respectively. GS activity was assayed using the hydroxylamine-dependent synthetase method [30] in 50 mM Tris–HCl (pH 7.5), 5 mM ATP, 125 mM Na-Glu, 40 mM $MgSO_4$ and 5 mM NH_2OH. The activity due to each isoform was estimated as the area under the curve of their corresponding peaks.

4.7. Total RNA extraction, cDNA synthesis and semi-quantitative PCR

Total RNA was extracted from 100 mg of frozen leaves with Concert Plant RNA Reagent (Invitrogen). One microgram of DNAse treated total RNA was used for cDNA synthesis by reverse transcription with RT Superscript II (Invitrogen) following the manufacturer's protocol. cDNA samples were used as templates to quantify the expression levels of target genes. Semi-quantitative PCR analysis was carried out using gene-specific primers designed to amplify specifically as follows: for *Gs2* group they were 5′-ATTTCGAAGCCAGTGGAG-3′ and 5′-GCACTTGTGCAGTGACCTTG-3′, beginning at 283 and 535 bp respectively, relative to *Gs2a* sequence For *Gs1* group they were 5′-CAACCCTGATGTTGCCAAG-3′ and 5′-GTAGGCGGCGATGTGCT-3′, beginning at 345 and 852 respectively, relative to *Gs1c* sequence. For *Gsr* group they were 5′-CAAGATCTTCAACACCCCGA-3′ and 5′-GTAGGCGGCGATGTGCT-3′ in position 418 and 937 respectively relative to *Gsr1* sequence and for *Gse* group they were 5′-CGAGGT-CAAGGCTGAAGAAC-3′ and 5′-GTAGGCGGCGATGTGCT-3′ in position 467 and 968 respectively relative to *Gse2* sequence. Constitutive expression of wheat translation elongation factor 1-α-subunit (*Tef1*) mRNA (GenBank accession nos. M90077) was determined to confirm the equality of molecular copies and as internal standard. Samples were denatured at 94 °C for 2 min, followed by 15–30 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s). The levels of amplified products were determined at several cycle intervals to ensure that samples were analysed during the exponential phase of amplification and each PCR product was sequenced to corroborate the specific amplification of the desired gene. Reactions carried out in the absence of reverse transcriptase were used to control the presence of contaminating DNA.

4.8. Band quantification

The integrated density values of the bands from protein and PCR product gels were analysed using ImageJ 1.38x software, free available at http://rsb.nih.gov/ij/index.html.

4.9. Statistical analysis

Analysis of variance and regression analysis of the data were performed using Statgraphics (R) software.

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Appendix. Supplementary material

Alignment of *Gs1a, Gs1b, Gs1c, Gse1, Gse2, Gsr1, Gsr2, Gs2a, Gs2b and Gs2c* sequences and primers location: FW Gs2 and complementary sequence to RV Gs2, in position 283 and 535 bp both relative to *Gs2a*; FW Gs1 in position 345 bp relative to *Gs1c*, FW Gsr in position 418 relative to *Gsr1*, FW Gse in position 467 relative to *Gse2* and the complementary sequence to the primer used to amplified *Gs1, Gsr and Gse* genes in position 852 relative to *Gs1c*. Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.plaphy.2009.01.003.

References

[1] A.J. Barneix, H.F. Causin, The central role of amino acids on nitrogen utilization and plant growth, J. Plant Physiol. (1996) 358–362.
[2] T.W. Becker, M. Caboche, E. Carrayol, B. Hirel, Nucleotide sequence of a tobacco cDNA encoding plastidic glutamine synthetase and light inducibility, organ specificity and diurnal rhythmicity in the expression of the corresponding genes of tobacco and tomato, Plant Mol. Biol. 3 (1992) 367–379.
[3] M.M. Bradford, A rapid and sensitive method for utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[4] N. Brugière, F. Dubois, A.M. Limami, M. Lelandais, Y. Roux, R.S. Sangwan, B. Hirel, Glutamine synthetase in the phloem plays a major role in controlling proline production, Plant Cell 10 (1999) 1995–2012.

[5] N. Brugière, F. Dubois, C. Masclaux-Daubresse, R.S. Sangwan, B. Hirel, Immunolocalization of glutamine synthetase in senescing tobacco (*Nicotiana tabacum* L.) leaves suggests that ammonia assimilation is progressively shifted to the mesophyll cytosol, Planta 4 (2000) 519–527.
[6] F.R. Cantón, A. Garcia-Gutierrez, R. Crespillo, F.M. Cánovas, High-level expression of *Pinus sylvestris* glutamine synthetase in *Escherichia coli*. Production of polyclonal antibodies against the recombinant protein and expression studies in pine seedlings, FEBS Lett. (1996) 205–210.
[7] C. Caputo, A.J. Barneix, Export of amino acids to the phloem in relation to N supply in wheat, Physiol. Plant 101 (1997) 853–860.
[8] M. Cren, B. Hirel, Glutamine synthetase in higher plants regulation of gene and protein expression from the organ to the cell, Plant Cell Physiol. 12 (1999) 1187–1193.
[9] M.V. Criado, I.N. Roberts, M. Echeverria, A.J. Barneix, Plant growth regulators and induction of leaf senescence in nitrogen-deprived wheat plants, J. Plant Growth Regul. 26 (2007) 301–307.
[10] J. Edwards, E. Walker, G. Coruzzi, Cell-Specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase, Proc. Natl. Acad. Sci. U.S.A. 9 (1990) 3459–3463.
[11] U. Feller, A.M. Fischer, Nitrogen metabolism in senescing leaves, Crit. Rev. Plant Sci. 3 (1994) 241–273.
[12] M.S. Fernandes, R.O.P. Rossiello, Mineral nitrogen in plant physiology and plant nutrition, Crit. Rev. Plant Sci. 2 (1995) 111–148.
[13] D.Z. Habash, A.J. Massiah, H.L. Rong, R.M. Wallsgrove, R. Leigh, The role of cytosolic glutamine synthetase in wheat, Ann. Appl. Biol (2001) 83–89.
[14] T.A. Hall, BioEdit a user-friendly biological sequence alignment editor and analysis programme for Windows 95/98/NT, Nucleic Acids Symp. Ser. 41 (1999) 95–98.
[15] H. Hayashi, M. Chino, Chemical composition of phloem sap from the upper most internode of the rice plant, Plant Cell Physiol. 31 (1990) 247–251.
[16] B. Hirel, P.J. Lea, Ammonium assimilation, in: P.J. Lea, J.F. Morot-Gaudry (Eds.), Plant Nitrogen, INRA Springer-Verlag Inc., Berlin, 2001, pp. 79–99.
[17] B. Hirel, B. Andrieu, M.-H. Valadier, S. Renard, I. Quillere, M. Chelle, B. Pommel, C. Fournier, J.-L. Drouet, Physiology of maize II: identification of physiological markers representative of the nitrogen status of maize (*Zea mays*) leaves during grain filling, Physiol. Plant 2 (2005) 178–188.
[18] B. Hirel, J. Le Gouis, B. Ney, A. Gallais, The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches, J. Exp. Bot. 9 (2007) 2369–2387.
[19] D.R. Hoagland, D.I. Arnon, The water culture method for growing plants without soil, Calif. Agric. Exp. Sta. Circ. 347 (1950) 1–39.
[20] K. Ishiyama, E. Inoue, M. Tabuchi, T. Yamaya, H. Takahashi, Biochemical background and compartmentalized functions of cytosolic glutamine synthetase for active ammonium assimilation in rice roots, Plant Cell Physiol. 11 (2004) 1640–1647.
[21] K. Ishiyama, E. Inoue, A. Watanabe-Takahashi, M. Obara, T. Yamaya, H. Takahashi, Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in Arabidopsis, J. Biol. Chem. 16 (2004) 16598–16605.
[22] W. Jordi, A. Schapendonk, E. Davelaar, G.M. Stoopen, C.S. Pot, R. De Visser, J.A. Van Rhijn, S. Gan, R.M. Amasino, Increased cytokinin levels in transgenic P_{SAG12}-*IPT* tobacco plants have large direct and indirect effects on leaf senescence, photosyntesis and N partitioning, Plant Cell Environ. (2000) 279–289.
[23] T. Kichey, J. Le Gouis, B. Sangwan, B. Hirel, F. Dubois, Changes in the cellular and subcellular localization of glutamine synthetase and glutamate dehydrogenase during flag leaf senescence in wheat (*Triticum aestivum* L.), Plant Cell Physiol. 6 (2005) 964–974.
[24] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[25] H.-M. Lam, K.T. Coschigano, C. Schultz, R. Melo-Oliveira, G. Tjaden, I.C. Oliveira, N. Ngai, M.-H. Hsieh, G. Coruzzi, Use of Arabidopsis mutants and genes to study amide amino acid síntesis, Plant Cell 7 (1995) 887–898.
[26] D.A. Lightfoot, N.K. Green, J.V. Cullimore, The chloroplast-located glutamine synthetase of *Phaseolus vulgaris* L.: nucleotide sequence, expression in different organs and uptake into isolated chloroplasts, Plant Mol. Biol. 2 (1988) 191–202.
[27] L. Lima, A. Seabra, P. Melo, J. Cullimore, H. Carvalho, Post-translational regulation of cytosolic glutamine synthetase of *Medicago truncatula*, J. Exp. Bot. 57 (2006) 2751–2761.
[28] A. Martin, J. Lee, T. Kichey, D. Gerentes, M. Zivy, C. Tatout, F. Dubois, T. Balliau, B. Valot, M. Davanture, T. Terce-Laforgue, I. Quillere, M. Coque, A. Gallais, M.-B. Gonzalez-Moro, L. Bethencourt, D.Z. Habash, P.J. Lea, A. Charcosset, P. Perez, A. Murigneux, H. Sakakibara, K.J. Edwards, B. Hirel, Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production, Plant Cell 11 (2006) 3252–3274.
[29] B.J. Miflin, D.Z. Habash, The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops, J. Exp. Bot. 370 (2002) 979–987.
[30] T. Nesselhut, G. Harnischfeger, Characterization of glutamine synthetase from *Beta vulgaris*, Physiol. Plant 51 (1981) 329–334.
[31] R.J. Simpson, M.J. Dalling, Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L.), Planta 151 (1981) 447–456.
[32] M. Suárez, C. Avila, F. Gallardo, F. Cantón, A. García-Gutiérrez, M. Claros, F. Cánovas, Molecular and enzymatic analysis of ammonium assimilation in woody plants, J. Exp. Bot. 53 (2002) 891–904.
[33] M. Tabuchi, K. Sugiyama, K. Ishiyama, E. Inoue, T. Sato, H. Takahashi, T. Yamaya, Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase1;1, Plant J. 5 (2005) 641–651.
[34] T. Terce-Laforgue, G. Mack, B. Hirel, New insights towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (*Nicotiana tabacum*) plants grown under different nitrogen regimes, Physiol. Plant 120 (1994) 220–228.
[35] H. Towbin, T. Staehlin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 4350–4353.
[36] A. Watanabe, N. Takagi, H. Hayashi, M. Chino, A. Watanabe, Internal Gln/Glu ratio as a potential regulatory parameter for the expression of a cytosolic glutamine synthetase gene of radish in cultured cells, Plant Cell Physiol. 9 (1997) 1000–1026.
[37] E.W. Yemm, E.C. Cocking, The determination of amino acids with ninhydrin, Analyst (1955) 209–213.
[38] E.W. Yemm, A.J. Willis, The estimation of carbohydrates in plant extracts by anthrone, J. Biochem. (1954) 508–514.