



**HAL**  
open science

## Metabolic profiling of maize mutants deficient for two glutamine synthetase isoenzymes using <sup>1</sup>H-NMR-based metabolomics

Caroline Broyart, Jean-Xavier J.-X. Fontaine, Roland Molinié, Dominique Cailleu, Thérèse Tercé-Laforgue, Frédéric Dubois, Bertrand Hirel, François Mesnard

### ► To cite this version:

Caroline Broyart, Jean-Xavier J.-X. Fontaine, Roland Molinié, Dominique Cailleu, Thérèse Tercé-Laforgue, et al.. Metabolic profiling of maize mutants deficient for two glutamine synthetase isoenzymes using <sup>1</sup>H-NMR-based metabolomics. *Phytochemical Analysis*, 2010, 21 (1), pp.102-109. 10.1002/pca.1177 . hal-01203907

**HAL Id: hal-01203907**

**<https://hal.science/hal-01203907v1>**

Submitted on 30 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Metabolic Profiling of Maize Mutants Deficient for Two Glutamine Synthetase Isoenzymes Using <sup>1</sup>H-NMR-based Metabolomics<sup>†</sup>

Caroline Broyart,<sup>a,b</sup> Jean-Xavier Fontaine,<sup>a</sup> Roland Molinié,<sup>a</sup>  
Dominique Cailleu,<sup>c</sup> Thérèse Tercé-Laforgue,<sup>b</sup> Frédéric Dubois,<sup>a</sup>  
Bertrand Hirel<sup>b</sup> and François Mesnard<sup>a\*</sup>

## ABSTRACT:

**Introduction** – Maize mutants deficient for the expression of two genes encoding cytosolic glutamine synthetase (GS) isoenzymes GS1.3 and GS1.4 displayed reduced kernel number and kernel size, respectively, the effect of the mutation being cumulative in the double mutant. However, at maturity, shoot biomass production was not modified in all the mutants, indicating that the reaction catalysed by the enzyme is specifically involved in the control of grain yield.

**Objective** – To examine the physiological impact of the GS mutations on the leaf metabolic profile during the kernel filling period, during which nitrogen is remobilised from the shoots to be further exported to the kernels.

**Methodology** – An <sup>1</sup>H-NMR spectroscopy metabolomic was applied to the investigation of metabolic change of the *gln1.3*, *gln1.4* and *gln1.3/1.4* double mutant.

**Results** – In the three GS mutants, an increase in the amount of several N-containing metabolites such as asparagine, alanine, threonine and phosphatidylcholine was observed whatever the level of nitrogen fertilisation. In addition, we found an accumulation of phenylalanine and tyrosine, two metabolites involved in the primary steps of the phenylpropanoid pathway.

**Conclusion** – Changes in the metabolic profile of the GS mutants suggest that, when cytosolic GS activity is strongly reduced, either alternative metabolic pathways participate in the reassimilation of ammonium released during leaf protein remobilisation or that premature leaf senescence is induced when kernel set and kernel filling are affected. The accumulation of phenylalanine and tyrosine in the mutant plants indicates that lignin biosynthesis is altered, thus possibly affecting ear development. Copyright © 2009 John Wiley & Sons, Ltd.

**Keywords:** glutamine synthetase; *Zea mays*; metabolomics; mutants; nuclear magnetic resonance spectroscopy

## Introduction

Since the green revolution, nitrogen (N) fertilisation has been increasingly used in agriculture to increase yield in cereals and other coarse grain crops. Nevertheless, both to avoid pollution by nitrate and to maintain a sufficient profit margin, farmers have to reduce the use of N fertiliser. This can be achieved by developing crop varieties that have a better nitrogen use efficiency (NUE). Thus, a better knowledge of the physiological basis and genetic basis of NUE is necessary in order to improve N uptake and utilisation efficiency either through conventional breeding or genetic engineering (Hirel *et al.*, 2007). Over the last decade, it has been put forward on a regular basis that the reaction catalysed by the enzyme glutamine synthetase (GS; EC 6.3.1.2) is an important step in the control of plant productivity (Andrews *et al.*, 2004; Gallais and Hirel, 2004; Hirel *et al.*, 2007; Bernard and Habash, 2009). The GS enzyme catalyses an ATP-dependent conversion of glutamate to glutamine using ammonium as a substrate. Ammonium can be directly taken up from the soil or originates from a variety of physiological processes such as nitrate reduction, photorespiration, amino acid interconversion and leaf protein remobilisation (Ireland and Lea, 1999). In higher plants, GS is represented by two isoenzymes, located either in the

cytosol (GS1) or in the plastids (GS2) (Hirel and Lea, 2001). GS2 is encoded by a single nuclear gene per haploid genome, whereas GS1 is encoded by a small multigene family containing up to five genes depending on the plant species examined (Cren and Hirel, 1999). For example there are five genes encoding GS1 in

\* Correspondence to: F. Mesnard, Laboratoire de Phytotechnologie EA3900-BioPI, Faculté de Pharmacie, 1, rue des Louvels 80037 Amiens cedex 1, France. E-mail: Francois.mesnard@u-picardie.fr

<sup>a</sup> EA 3900-BioPI Biologie des Plantes et Contrôle des Insectes Ravageurs, Faculté de Pharmacie, 1, rue des Louvels et Faculté des Sciences, 33, rue Saint Leu, 80037 Amiens cedex 1, France

<sup>b</sup> Laboratoire de la Nutrition Azotée des Plantes, UMR 511, INRA, Centre de Versailles, R.D. 10, 78026 Versailles cedex, France

<sup>c</sup> Plateforme Analytique, Bâtiment Serres-Transfert, rue de Mai/rue Dallery 80039 Amiens cedex 1, France

<sup>†</sup> This article is published in *Phytochemical Analysis* as a special issue on Metabolomics in Plant and Herbal Medicine Research, edited by Young Hae Choi, Hye Kyong Kim and Robert Verpoorte, all from Leiden University in the Netherlands.

Arabidopsis (Ishiyama *et al.*, 2004) and maize (Hirel *et al.*, 2005), three genes encoding GS1 in wheat (Habash *et al.*, 2007) and rice (Tabuchi *et al.*, 2005), and two genes encoding GS1 in *Medicago truncatula* (Cavalho *et al.*, 1997) and potato (Teixeira *et al.*, 2005). Among these different GS1 isoforms, one is localised in the phloem whatever the plant examined (Tobin and Yamaya, 2001; Dubois *et al.*, 1996; Brugière *et al.*, 1999; Teixeira *et al.*, 2005; Sakurai *et al.*, 1996; Tabuchi *et al.*, 2005; Kichey *et al.*, 2005; Martin *et al.*, 2006; Bernard *et al.*, 2008). The phloem GS1 isoform is likely to be involved in the synthesis of glutamine used for long-distance transport of N-containing molecules. Another cytosolic GS isoform, which is usually also highly expressed in root tissues, is induced in the cytosol of the leaf parenchyma cells of C3 plants during leaf senescence (Brugière *et al.*, 2000; Kichey *et al.*, 2005; Bernard *et al.*, 2008). This isoform of GS1 is probably involved in the recycling of ammonium released during leaf protein remobilisation from source to sink organs (Bernard and Habash, 2009; Masclaux *et al.*, 2001). In a C4 plant such as maize, two of the five genes encoding a GS1 (*Gln1-3* and *Gln1-4*) are highly expressed in most plant organs regardless of leaf age and environmental conditions (Sakakibara *et al.*, 1992; Li *et al.*, 1993; Limami *et al.*, 2001; Hirel *et al.*, 2005). Recently, it has been suggested that *Gln1-4* is involved in the reassimilation of ammonium released during leaf protein degradation, whereas *Gln1-3* plays a house-keeping function throughout plant growth and development (Limami *et al.*, 2002; Hirel *et al.*, 2005). Moreover, the importance of *Gln1-3* and *Gln1-4* for maize productivity has been recently emphasised using both forward and reverse genetic approaches leading Martin *et al.* (2006) to conclude that GS1 plays a major role in the control of kernel yield and its components but is not involved in the control of vegetative plant biomass production (Martin *et al.*, 2006; Hirel *et al.*, 2007). Preliminary physiological analysis of maize *gln1.3*, *gln1.4* and *gln1.3/gln1.4* double mutants showed that N metabolism is altered as the result of reduced GS1 activity in leaves (Martin *et al.*, 2006). To further investigate both the physiological and developmental impact of the *gln1.3*, *gln1.4* and *gln1.3/gln1.4* knockout mutations on plant performance, we performed a metabolomic profiling study using <sup>1</sup>H-nuclear magnetic resonance (NMR) spectroscopy followed by multivariate data analysis. This technique, which is a non-targeted analytical method, is able to provide a wide range of information about the metabolite content in different organs and thus on the overall plant metabolic status in a relatively short period of time on a large number of samples (Verpoorte *et al.*, 2007).

## Experimental

### Plant material

The isolation and characterisation of maize *gln1-3*-, *gln1-4*- and *gln1-3/1-4*- deficient mutants was previously described by Martin *et al.* (2006). Maize mutants and wild-type (WT) plants were grown under suboptimal N conditions (N<sup>+</sup>) or under N-limiting conditions (N<sup>-</sup>) using the growth conditions used by Martin *et al.* (2006). The leaf below the ear was harvested at 55 days after silking. The leaf below the ear was selected since it has been shown to provide a good indication of the source-sink transition during grain filling which corresponds to the translocation of N and carbon assimilates from the leaf to the developing ear (Hirel *et al.*, 2005; Martin *et al.*, 2005, 2006). Leaf samples were harvested between 9 and 12 a.m. and frozen in liquid N<sub>2</sub>, ground to a homogenous powder using a grinder (MM301, Retsch, Germany), stored at -80°C and freeze-dried. These leaf samples used for NMR analysis were the same as those used for the molecular and physiological characterisation of the GS1 deficient mutants of maize (Martin *et al.*, 2006).

### Metabolite extraction

Fifty milligrams of freeze-dried material were transferred to a 2 mL micro-tube containing 1.5 mL of extraction buffer composed of 750 µL of CH<sub>3</sub>OH-*d*<sub>4</sub> and 750 µL of D<sub>2</sub>O (KH<sub>2</sub>PO<sub>4</sub> buffer, pH 6) containing trimethyl silyl propionic acid sodium salt (TMSP-*d*<sub>4</sub>). The tube was shaken at room temperature for 1 min using a vortex homogeniser, then sonicated for 25 min, and finally centrifuged at 14000 rpm at room temperature for 30 min. Six hundred microlitres of supernatant were then transferred into a 5 mm NMR tube.

### NMR spectra measurements

All spectra (<sup>1</sup>H-NMR, *J*-resolved, TOCSY and HSQC) were recorded at 300 K on a Bruker Avance DMX500 spectrometer operating at 500.13 MHz for <sup>1</sup>H using a multinuclear broadband TXI 5 mm probe. CH<sub>3</sub>OH-*d*<sub>4</sub> was used as the internal lock. <sup>1</sup>H NMR spectra consisted of 128 scans of 32 K data points with a spectral width of 5208 Hz and a water suppression pulse sequence with a relaxation delay of 2 s. The resulting <sup>1</sup>H spectra were manually phased and baseline-corrected, and calibrated to TMSP at 0.0 ppm, all using XWIN NMR (version 3.3, Bruker). The FID was multiplied by an exponential weighing function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation. Two-dimensional *J*-resolved NMR spectra were acquired with 1.0 s relaxation delay using 64 scans per 64 increments that were collected into 16 K data points, using spectral widths of 5208 Hz in F2 and 50 Hz in F1. *J*-resolved spectra were tilted by 45° and symmetrised about F1. The TOCSY spectra were acquired using 24 scans per 512 increments that were collected into 4 K data points, using spectral widths of 5208 Hz in both dimensions. A 2.0 s relaxation delay and a mixing time of 60 ms were employed. The HSQC NMR spectra were acquired with 1.0 s relaxation delay using 128 scans per 256 increments that were collected into 2 K data points, using spectral widths of 5208 Hz in F2 and 22638 Hz in F1. All 2D spectrum were calibrated at 0.0 ppm to TMSP.

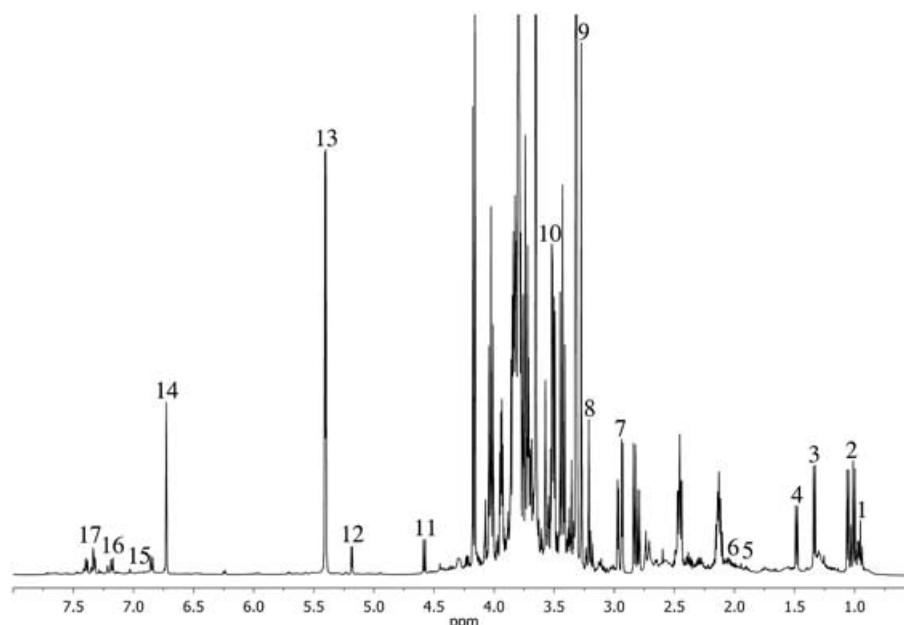
### Data analysis

The <sup>1</sup>H-NMR spectra were automatically reduced to ASCII files using MestReNova (version 5.2.5, Mestrelab Research, Santiago de Compostella, Spain). The region between δ 4.85 and δ 4.75 was removed from the analysis because of the residual signal of water. The residual proton signals corresponding to CH<sub>3</sub>OH-*d*<sub>4</sub> (δ 3.33 to δ 3.25) were also removed. Spectral intensities were scaled to the total area and reduced to integrated regions or 'buckets' of equal width (0.04 ppm) corresponding to the region of δ 10.0 to δ 0.3. The generated ASCII file was imported into Microsoft EXCEL for the addition of labels. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed with the SIMCA-P software (version 11.0, Umetrics, Umeå, Sweden). The scaling method for PCA was Pareto and for PLS-DA the unit-variance method was used. Each signal identified by PLS-DA as discriminant was integrated using MestReNova. The resulting area was used to test the significance of the differences observed between WT and mutants by a student test using the Excel software (Microsoft). The PLS-DA model was validated using the permutation method through 20 applications.

## Results and Discussion

### Metabolites identification by one- and two-dimensional NMR spectra

The <sup>1</sup>H-NMR spectra of the *gln1-3*, *gln1-4* and *gln1-3/1-4* mutants leaves were compared with those of the WT. A number of primary and secondary metabolites were identified in the <sup>1</sup>H-NMR spectrum (Fig. 1). All metabolites were identified by comparison with chemical shifts of reference compounds. The complete assignments of the identified compounds signals were carried out by <sup>1</sup>H, *J*-resolved, TOCSY and HSQC spectra and with



**Figure 1.** Representative  $^1\text{H-NMR}$  spectrum in the range of  $\delta$  0.5 to  $\delta$  8.5. (1) Ileu and Leu, (2) Val, (3) Thr, (4) Ala, (5) GABA, (6) Pro, (7) Asn, (8) choline, (9) phosphatidylcholine, (10) Gly, (11)  $\beta$ -glucose, (12)  $\alpha$ -glucose, (13) sucrose, (14) fumaric acid, (15) chlorogenic acid, (16) Tyr, (17) Phe.

the comparison of reference compounds and previously reported data (Manetti *et al.*, 2006; Castro *et al.*, 2008; Piccioni *et al.*, 2009). It was possible to identify the amino acids such as alanine (Ala), asparagine (Asp), glycine (Gly), isoleucine (Ileu), leucine (Leu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr) and valine (Val) as well as  $\gamma$ -aminobutyric acid (GABA). Organic acids such as fumaric acid and carbohydrates such as sucrose and glucose were also identified due to their anomeric signals and by comparison with pure compounds. By analysis of  $J$ -resolved spectra, the signals from the two *trans* olefinic protons of chlorogenic acid (H-7 at  $\delta$  7.60, H-8 at  $\delta$  6.35) were characterised as described previously (Leiss *et al.*, 2009). In the  $^1\text{H-NMR}$  spectrum

the chemical shift at  $\delta$  1.34 was attributed to Thr with the help of the TOCSY spectrum. In addition, the chemical shift of the two protons at  $\delta$  3.50 and at  $\delta$  4.22 was the  $\alpha$ -CH and  $\beta$ -CH, respectively attributed to Thr. Moreover, phospholipids such as phosphatidylcholine and nitrogenous compounds like choline were assigned with the help of HSQC.  $J$ -resolved and TOCSY spectra allowed the differentiation of Ileu and Leu. With the help of the chemical shift at  $\delta$  3.56 and the doublet at  $\delta$  1.02 ( $J$ -resolved spectra), Ileu was identified with confidence. Leu was identified by the chemical shift at  $\delta$  0.96,  $\delta$  9.9 thanks to  $J$ -resolved spectra and with the chemical shift at  $\delta$  3.65 thanks to TOCSY spectra (Table 1).

**Table 1.** Resonance assignments of metabolites identified in NMR spectra of maize with chemical shifts ( $\delta$ ) and coupling constants (Hz) ( $\text{CH}_3\text{OH-}d_4\text{-KH}_2\text{PO}_4$  in  $\text{D}_2\text{O}$ ; pH 6.0)

Compound	Chemical shifts (ppm) and coupling constants (Hz)
Ala	1.48 ( $\beta$ - $\text{CH}_3$ , d, $J = 7.2$ Hz)
$\alpha$ -Aminobutyric acid (GABA)	1.90 ( $\beta$ - $\text{CH}_2$ , m), 2.36 ( $\alpha$ - $\text{CH}_2$ , t, $J = 7.2$ Hz), 3.00 ( $\gamma$ - $\text{CH}_2$ , t, $J = 7.5$ Hz)
Asn	2.82 ( $\beta$ - $\text{CH}_2$ , dd, $J = 17.0, 8.1$ Hz), 2.95 ( $\beta'$ - $\text{CH}_2$ , dd, $J = 17.0, 4.0$ Hz), 3.94 ( $\alpha$ -CH, dd, $J = 8.2, 4.0$ Hz)
Chlorogenic acid	6.35 (H-8, d, $J = 16.0$ Hz), 6.87 (H-5, d, $J = 8.2$ Hz), 7.05 (H-6, dd, $J = 8.3, 1.9$ Hz), 7.13 (H-2, d, $J = 1.9$ Hz), 7.60 (H-7, d, $J = 15.9$ Hz)
Choline	3.21 ( $\text{CH}_3$ , s)
Fumaric acid	6.73 (H-2 and H-3, s)
$\beta$ -Glucose	4.57 (H-1, d, $J = 7.9$ Hz)
$\alpha$ -Glucose	5.17 (H-1, d, $J = 3.8$ Hz)
Gly	3.57 ( $\alpha$ - $\text{CH}_2$ , s)
Ileu	0.95 ( $\delta$ - $\text{CH}_3$ , t, $J = 7.4$ Hz), 1.02 ( $\gamma$ - $\text{CH}_3$ d, $J = 7.0$ Hz), 3.56 ( $\alpha$ -CH, d, $J = 4.0$ Hz)
Leu	0.96 ( $\delta$ - $\text{CH}_3$ , d, $J = 6.6$ Hz), 0.98 ( $\delta'$ - $\text{CH}_3$ , d, $J = 6.6$ Hz), 3.65 ( $\alpha$ -CH, m)
Phe	7.3 to 7.4 (H-2, H-3, H-4, H-5, and H-6, m)
Phosphatidylcholine	3.27 ( $\text{CH}_3$ , s)
Pro	2.00 ( $\gamma$ - $\text{CH}_2$ , m), 4.06 ( $\alpha$ -CH, dd, $J = 8.7, 6.3$ Hz)
Sucrose	4.16 (H-3 of fructose moiety, d, $J = 8.6$ Hz), 5.40 (H-1 of glucose moiety, d, $J = 3.8$ Hz)
Thr	1.34 ( $\gamma$ - $\text{CH}_3$ , d, $J = 6.6$ Hz), 3.50 ( $\alpha$ -CH, d, $J = 5.2$ Hz), 4.22 ( $\beta$ -CH, m)
Tyr	6.85 (H-2 and H-6, m), 7.18 (H-3 and H-5, ring, m)
Val	1.00 ( $\gamma$ - $\text{CH}_3$ , d, $J = 7.0$ Hz), 1.05 ( $\gamma'$ - $\text{CH}_3$ , d, $J = 7.0$ Hz)

## Data analysis

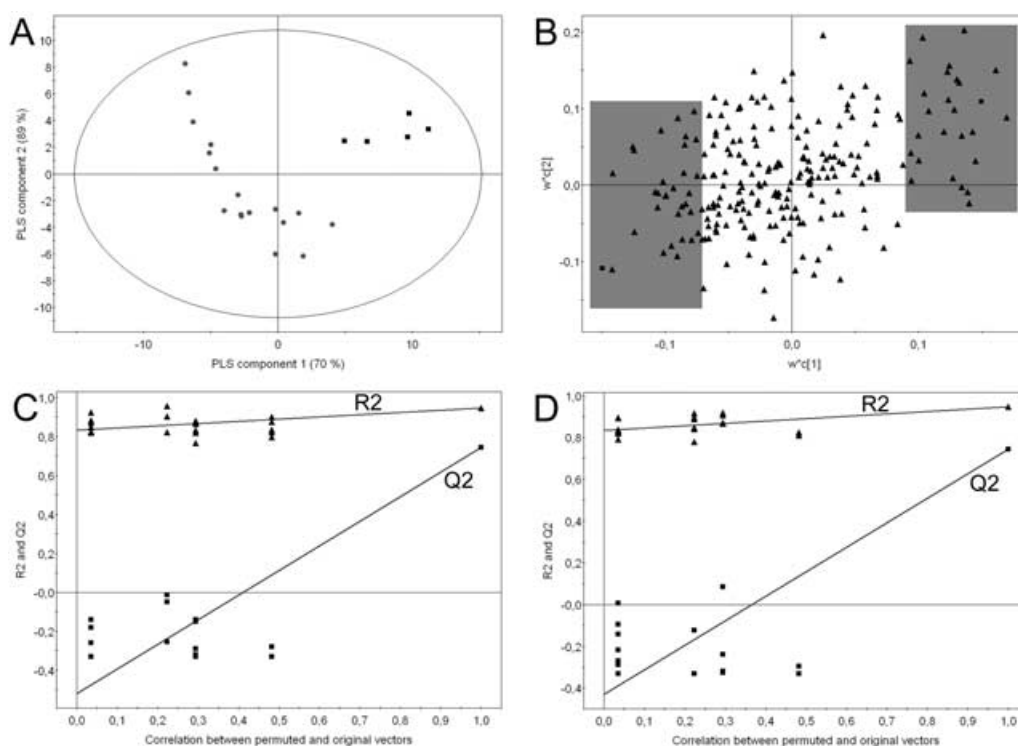
In order to select the resonances corresponding to metabolite content that was altered in the *gln1.3*, *gln1.4* and *gln1.3/1.4* mutants, multivariate data analysis were performed. Indeed, metabolomics experiments produce a large set of data. For a first overview of metabolomic changes, multivariate analysis can deal with a large data set by reducing the dimensionality of the  $^1\text{H-NMR}$  data. Principal component analysis is an unsupervised method which reduces the dimensionality of a given data set by producing new linear combinations of the original variables. PCA allows the visualisation of trends, clustering, similarity/dissimilarity among the samples, thus giving a macroscopic metabolic differentiation within evaluated samples (Leiss *et al.*, 2009). For this, raw NMR data have to be bucketed in order to align the signals and avoiding possible fluctuations. The signals in the region between  $\delta 0.3$  and  $\delta 10.0$  were bucketed every 0.04 ppm, generating 242 discrete chemical shift regions to produce a series of sequentially integrated regions. After residual signals from water and methanol- $d_4$  were suppressed, 235 variables were submitted to PCA analysis and thus reduced to a few principal components. This explains the variation among the samples in terms of metabolic changes. However, it was not possible to discriminate correctly the three GS1 mutants from the WT (data not shown), indicating that the biological variations within the samples of the same groups could be higher than between groups. In this case, a supervised multivariate data analysis using covariance is required to try to associate a group of metabolites to the mutation. Partial least square discriminant analysis corresponds to

such a supervised method. In contrast to PCA, which only uses the information of the metabolomic matrix, PLS-DA also takes into account the matrix of the plant type. PLS-DA uses a discrete class matrix (in this case 0 for the GS1 mutants and 1 for WT). The separation of PLS-DA is achieved by the covariance of the two datasets. With PLS-DA, the separation between WT and the three maize GS1 mutants was considerably improved (Fig. 2A). The permutation test through 20 applications for the wild-type group (Fig. 2C) and mutant group (Fig. 2D) validated the PLS-DA model. Indeed, all Q2 values of permuted Y vectors were lower than the original ones and the regression of Q2 lines intersected the y-axis at below zero (Fig. 2C and D), indicating that the model was valid (Leiss *et al.*, 2009 citing Eriksson *et al.*, 2001).

In accordance with the loading plot (Fig. 2B), Asp, sucrose, glucose and Phe were assigned to the maize GS1 mutants by comparison with the WT. The WT was characterised by a high content of Ileu, GABA and Pro. The *t*-test applied to the data defined the statistically significant variables found between the mutants and the WT. From the 235  $^1\text{H-NMR}$  variables 49 were significantly different ( $p < 0.05$ ). Among these variables, signals for Asp, sucrose, glucose, Tyr, chlorogenic acid, Ileu GABA and Pro, which were identified by PLS-DA, were confirmed as differentiating significantly the two groups of samples.

## Metabolite ratio analysis

Since overlapping can occur in 1D- $^1\text{H-NMR}$  spectra, data obtained by buckets for PLS-DA analysis can be biased. Therefore,



**Figure 2.** (A) Score plot of PLS-DA of crude extracts from maize leaves of WT ( $v$ ) and cytosolic GS mutants ( $\lambda$ ). (B) Loading plot of PLS-DA in leaves using two classes (class 0, WT; class 1, cytosolic GS mutant). Each triangle ( $\sigma$ ) represents the chemical shifts from bucketed  $^1\text{H-NMR}$  data. The grey frames show the discriminant chemical shifts of Asn,  $\alpha$ -glucose,  $\beta$ -glucose, sucrose, tyrosine and phenylalanine contained in cytosolic GS mutant (on the bottom left) and Ileu GABA and Pro contained in WT (on the top right). (C) and (D) represent permutation validation (20 permutations with three components) plot for the mutant group (C) and the susceptible group (D) of PLS-DA based on  $^1\text{H-NMR}$  spectra. In this validation plot R2 stands for variance and Q2 stands for predictive ability of the model. Leaf samples were harvested at maturity on plants grown under N suboptimal conditions.

J-resolved spectra were used to detect individualised signals of identified discriminant metabolites. A *t*-test was then applied to the area corresponding to these signals in order to determine statistically significant signals between the three GS1 mutants and the WT. The area was integrated and the spectra scaled to the total area or to TMSP area using MestReNova. These two integration methods gave the same results except for Thr, Ala, choline and phosphatidylcholine which were found to be discriminant with TMSP area correction. Most of the metabolites were confirmed as significantly different ( $p < 0.05$ ) in the two groups separately (i.e. N<sup>+</sup> or N<sup>-</sup>) or together (i.e. N<sup>+</sup> and N<sup>-</sup>) (Table 2). Those metabolites were visible on the loading plot, where plots corresponding to the area of Thr, Ala and phosphatidylcholine were found in the GS1 mutants (bottom left) and a plot corresponding to choline was found in the WT (top right), (Fig. 2B). In addition, the same scale of variation in metabolite content was observed between the mutants and the WT independently of the N fertilisation regime (N<sup>+</sup> or N<sup>-</sup>), except for fumaric acid, choline, Gly and Val, as reported in Table 2. Indeed fumaric acid, choline and Gly contents were higher in the WT in N<sup>+</sup>, whereas the Val content was lower in N<sup>-</sup>, which was the opposite in the mutants.

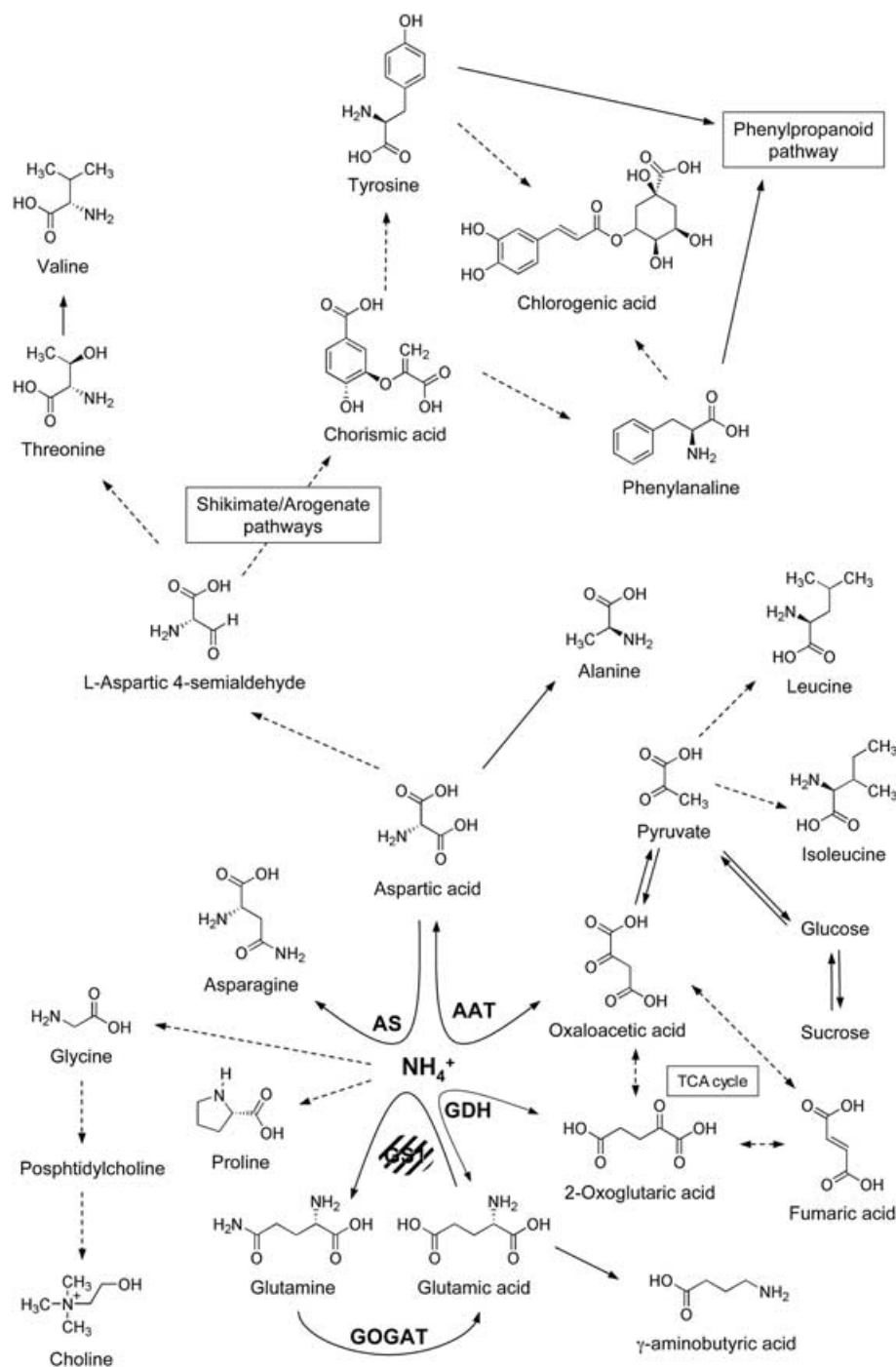
In this study, it was possible to observe a number of differences between the metabolic profiles of three different maize mutants deficient for cytosolic GS (namely *gln1.3*, *gln1.4* and *gln1.3/1.4* double mutant) and the WT. This indicates that, although the two isoforms GS1.3 and GS1.4 play a non-redundant function in the synthesis of Glu (Martin *et al.*, 2006), a number of metabolic functions associated with ammonium assimilation are commonly altered in the three GS mutants. These differences were assigned to particular metabolic pathways that were likely to play a compensatory role in the mutants, to circumvent the lack of GS activ-

ity in the cytosol. It has been generally observed that the enzyme activity remains relatively high in mature leaves of maize compared with younger leaves or other organs (Hirel *et al.*, 2005; Limami *et al.*, 2002; Cañas *et al.*, 2009). During grain filling, large amounts of ammonium are released in senescing leaves presumably as a result of chloroplastic protein hydrolysis (Hörtensteiner and Feller, 2002). The released ammonium is reassimilated into Gln through the reaction catalysed by GS. In the three GS mutants we observed a marked increase in Asp in agreement with previously published results (Martin *et al.*, 2006; Table 2). Under standard physiological conditions, Asn rarely accumulates, unless the plant is subjected to biotic or abiotic stresses. Large quantities of Asn can also be produced during seed germination or when reduced N is remobilised and transported from source to sink organs (Lea *et al.*, 2007). Interestingly, an accumulation of Asn was observed previously when GS activity was impaired in the nodules of temperate legumes (Carvalho *et al.*, 2003; Harrison *et al.*, 2003; Wong *et al.*, 2004). This result suggested that other enzymes such as asparagine synthetase (AS; EC 6.3.5.4) play an important role in by-passing the flux of reduced N to avoid the accumulation of ammonium that is a prerequisite for plant survival (Fig. 3). Because the number of kernels is reduced in the GS mutants, one cannot also rule out the possibility that premature leaf senescence accompanied by an increase in Asn, is occurring, thus mimicking the well-known effect of ear removal on plant development (Christensen *et al.*, 1981). In line with the former hypothesis, we observed an increase in a variety of nitrogenous compounds such as Ala, Phe, Thre, Tyr and phosphatidylcholine independently of the level of N fertilisation. The biosynthesis of all of these compounds is also a means for storing reduced N (Fig. 3). This observation fits with the current hypothesis that when other sources of reduced N than Gln (usually used for long

**Table 2.** Results (ratios from the WT to the three GS mutants) of selected compound signals from <sup>1</sup>H-NMR spectra obtained with extracts of maize leaves situated below the ear. Plants were grown either under high N (N<sup>+</sup>) or low N (N<sup>-</sup>) fertilisation conditions and the leaf material harvested 55 days after silking (Martin *et al.*, 2006)

ppm	Compound	Maturity		
		N <sup>+</sup> and N <sup>-</sup>	N <sup>+</sup>	N <sup>-</sup>
0.94–0.95	Ileu	1,41*	1,46**	1,25*
0.97–0.99	Leu	1,29	1,17	1,36**
0.99–1.01	Val	0,76	0,48*	1,68**
1.32–1.35	Thr	0,84	0,77	0,87**
1.45–1.50	Ala	0,76	0,71*	0,69
1.86–1.92	GABA	1,68*	1,39	1,86***
1.99–2.09	Pro	1,32***	1,37*	1,27*
2.92–2.98	Asn	0,48*	0,30*	1,00
3.20–3.22	Choline	1,21	1,32	0,78**
3.25–3.28	Phosphatidylcholine	0,60	0,59*	0,37**
3.54–3.59	Gly	1,09	1,54*	0,82*
4.56–4.60	β-Glucose	0,66**	0,69	0,61*
5.17–5.20	α-glucose	0,57**	0,63	0,49*
5.38–5.42	Sucrose	0,67*	0,76	0,56**
6.66–6.75	Fumaric acid	1,18	1,77*	0,88
6.86–6.90	Chlorogenic acid	1,01	1,32**	1,05
7.17–7.20	Tyr	0,48***	0,52**	0,87
7.31–7.45	Phe	0,43**	0,36*	0,94

\*\*\*\*\* Statistically significant difference at the 0.05, 0.01, 0.001 probability levels, respectively.



**Figure 3.** Metabolic pathways modified in the maize mutants deficient for cytosolic GS. Enzyme abbreviations: AAT, aspartate aminotransferase; AS, asparagine synthetase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS1, cytosolic glutamine synthetase.

distance transport in cereals (Mifflin and Habash, 2002), cannot be exported to the developing ear, both kernel set and kernel filling are altered (Martin *et al.*, 2006; Hirel *et al.*, 2007).

It is worth noting that although there is an accumulation of reduced N, as form of amino acids in the mutants, the level of Glu as not markedly modified. It is well established that plants maintain their relative Glu concentration within fairly narrow limits (Forde and Lea, 2007; Labboun *et al.*, 2009). One can hypothesise that the homeostasis of Glu is maintained in the GS mutants through the activity of metabolic pathways that are able to syn-

thesise or catabolise the amino acid. In the GS1 mutants, modifications in the ammonium assimilatory pathway could also involve the shikimate/arogenate pathway, as shown by the increase in their Phe and Tyr contents (Table 2). Deamination of Phe and Tyr, leading to the release of ammonium, is the first step of the phenylpropanoid pathway at the origin of lignin formation (Fig. 3). Lignification is a process during which N recycling and thus cytosolic GS play a major role (Whetten and Sederoff, 1995; Cánovas *et al.*, 2007). If the GS1–3 and GS1–4 isoenzymes are specifically expressed during ear development which requires

the synthesis of large amounts of lignin (Reeves, 1950), the ammonium released during this developmental process could not be reassimilated, thus perturbing its formation. The observation that the length of the ear in the GS1 mutants is shorter is in favour of this hypothesis (Martin et al., 2006). In conclusion, this detailed metabolic profiling study revealed that the role of GS1 in maize for the control of kernel yield may be more complex than originally described since ammonium can be released by a variety of metabolic pathways both in vegetative and reproductive organs (Hirel and Lea, 2001; Cañas et al., 2009). Further metabolomic and molecular genetic studies are therefore required to investigate the impact of a GS1 mutation in reproductive tissues and to determine if there is a regulatory control mechanism, mediated by the synthesis of Glu, and occurring between the leaves and the developing ear.

### Acknowledgements

The authors wish to thank Dr. Hye Kyong Kim, Dr. Young Hae Choi and Professor Robert Verpoorte from the University of Leiden for very helpful discussions.

### References

- Andrews M, Lea PJ, Raven JA, Lindsey K. 2004. Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. *Ann Appl Biol* **145**: 25–40.
- Bernard SM, Habash DZ. 2009. The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. *New Phytol* **182**: 608–620.
- Bernard SM, Möller ALB, Dionisio G, Kichey T, Jahn TP, Dubois F, Baudo M, Lopes MS, Tercé-Laforgue T, Foyer CH, Parry MAJ, Forde BG, Araus JL, Hirel B, Schjoerring JK, Habash DZ. 2008. Gene expression, cellular localisation and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.). *Plant Mol Biol* **67**: 89–105.
- Brugière N, Dubois F, Limami AM, Lelandais M, Roux Y, Sangwan RS, Hirel B. 1999. Glutamine synthetase in the phloem plays a major role in controlling proline production. *Plant Cell* **11**: 1995–2012.
- Brugière N, Dubois F, Masclaux C, Sangwan RS, Hirel B. 2000. Immunolocalization of glutamine synthetase in senescing tobacco (*Nicotiana tabacum* L.) leaves suggests that ammonia assimilation is progressively shifted to the mesophyll cytosol. *Planta* **211**: 519–527.
- Cañas RA, Quilleré I, Christ A, Hirel B. 2009. Nitrogen metabolism in the developing ear of maize (*Zea mays* L.): analysis of two lines contrasting in their mode of nitrogen management. *New Phytol* **184**: 340–352.
- Cánovas FM, Avila C, Cantón FR, Cañas RA, de la Torre F. 2007. Ammonium assimilation and amino acid metabolism in conifers. *J Exp Bot* **58**: 2307–2318.
- Carvalho H, Sunkel C, Salema R, Cullimore JV. 1997. Heteromeric assembly of the cytosolic glutamine synthetase polypeptides of *Medicago truncatula*: complementation of a *glnA* *Escherichia coli* mutant with a plant domain-swapped enzyme. *Plant Mol Biol* **35**: 623–632.
- Carvalho HG, Lopes-Cardoso IA, Lima LM, Melo PM, Cullimore JV. 2003. Nodule-specific modulation of glutamine synthetase in transgenic *Medicago truncatula* leads to inverse alterations in asparagine synthetase expression. *Plant Physiol* **133**: 243–252.
- Castro C, Motto M, Rossi V, Manetti C. 2008. Variation of metabolic profiles in developing maize kernels up- and down-regulated for the *hda101* gene. *J Exp Bot* **59**: 3913–3924.
- Cren M, Hirel B. 1999. Glutamine synthetase in higher plants regulation of gene and protein expression from the organ to the cell. *Plant Cell Physiol* **40**: 1187–1193.
- Christensen LE, Below FE, Hageman RH. 1981. The effects of ear removal on senescence and metabolism of maize 1. *Plant Physiol* **68**: 1180–1185.
- Dubois F, Brugière N, Sangwan RS, Hirel B. 1996. Localization of tobacco cytosolic glutamine synthetase enzymes and the corresponding transcripts shows organ- and cell-specific patterns of protein synthesis and gene expression. *Plant Mol Biol* **31**: 803–817.
- Eriksson L, Johansson E, Kettaneh-Wold N, Wold S. 2001. *Multi- and Megavariate Data Analysis: Principles and Applications*. Umetrics: Umeå.
- Forde BG, Lea PJ. 2007. Glutamate in plants: metabolism, regulation, and signalling. *J Exp Bot* **58**: 2339–2358.
- Gallais A, Hirel B. 2004. An approach to the genetics of nitrogen use efficiency in maize. *J Exp Bot* **55**: 295–306.
- Habash DZ, Bernard S, Schondelmaier J, Weyen J, Quarrie SA. 2007. The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield. *Theor Appl Genet* **114**: 403–419.
- Harrison J, Pou de Crescenzo MA, Sené O, Hirel B. 2003. Does lowering glutamine synthetase activity in nodules modify nitrogen metabolism and growth of *Lotus japonicus*? *Plant Physiol* **133**: 253–262.
- Hirel B, Lea P. 2001. Ammonium assimilation. In *Plant Nitrogen*, Lea PJ, Morot-Gaudry JF (eds). Springer: Berlin; 79–99.
- Hirel B, Martin A, Tercé-Laforgue T, Gonzalez-Moro MB, Estavillo JM. 2005. Physiology of maize I: A comprehensive and integrated view of nitrogen metabolism in a C4 plant. *Physiol Plant* **124**: 167–177.
- Hirel B, Le Gouis J, Ney B, Gallais A. 2007. 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* **58**: 2369–2387.
- Hörtensteiner S, Feller U. 2002. Nitrogen metabolism and remobilization during senescence. *J Exp Bot* **53**: 927–937.
- Ireland RJ, Lea PJ. 1999. The enzymes of glutamine, glutamate, asparagine and aspartate metabolism. In *Plant Amino Acids: Biochemistry and Biotechnology*, Singh BK (ed.). Marcel Dekker: New York; 49–110.
- Ishiyama K, Inoue E, Watanabe-Takahashi A, Obara M, Yamaya T, Takahashi H. 2004. Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in *Arabidopsis*. *J Biol Chem* **279**: 16598–16605.
- Kichey T, Le Gouis J, Sangwan B, Hirel B, Dubois F. 2005. 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* **46**: 964–974.
- Labboun S, Tercé-Laforgue T, Roscher A, Bedu M, Restivo FM, Velanis CN, Skopelitis DS, Moshou PN, Roubelakis KA, Suzuki A, Hirel B. 2009. Resolving the role of plant glutamate dehydrogenase: I. *In vivo* real time nuclear magnetic resonance spectroscopy experiments. *Plant Cell Physiol* **50**: 1761–1773.
- Lea P, Sodek L, Parry M, Shewry P, Halford N. 2007. Asparagine in plants. *Ann Appl Biol* **150**: 1–26.
- Leiss KA, Maltese F, Choi YH, Verpoorte R, Klinkhamer PGL. 2009. Identification of chlorogenic Acid as a resistance factor for thrips in chrysanthemum. *Plant Physiol* **150**: 1567–1575.
- Li MG, Villemur R, Hussey PJ, Silflow CD, Gantt JS, Snustad DP. 1993. Differential expression of six glutamine synthetase genes in *Zea mays*. *Plant Mol Biol* **23**: 401–407.
- Limami AM, Rouillon C, Glevarec G, Gallais A, Hirel B. 2002. Genetic and physiological analysis of germination efficiency in maize in relation to nitrogen metabolism reveals the importance of cytosolic glutamine synthetase. *Plant Physiol* **130**: 1860–1870.
- Manetti C, Bianchetti C, Casciani L, Castro C, Cocco MED, Miccheli A, Motto M, Conti F. 2006. A metabonomic study of transgenic maize (*Zea mays*) seeds revealed variations in osmolytes and branched amino acids. *J Exp Bot* **57**: 2613–2625.
- Martin A, Belastegui-Macadam X, Quilleré I, Floriot M, Valadier MH, Pommel B, Andrieu B, Donnison I, Hirel B. 2005. Nitrogen management and senescence in two maize hybrids differing in the persistence of leaf greenness. Agronomic, physiological and molecular aspects. *New Phytol.* **167**: 483–492.
- Martin A, Lee J, Kichey T, Gerentes D, Zivy M, Tatout C, Dubois F, Balliau T, Valot B, Davanture M, Tercé-Laforgue T, Quilleré I, Coque M, Gallais A, Gonzalez-Moro MB, Bethencourt L, Habash DZ, Lea PJ, Charcosset A, Perez P, Murigneux A, Sakakibara H, Edwards KJ, Hirel B. 2006. Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *Plant Cell* **18**: 3252–3274.
- Masclaux C, Quilleré I, Gallais A, Hirel B. 2001. The challenge of remobilization in plant nitrogen economy. A survey of physio-agronomic and molecular approaches. *Ann Appl Biol* **138**: 69–81.
- Mifflin BJ, Habash DZ. 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J Exp Bot* **53**: 979–987.



- Piccioni F, Capitani D, Zolla L, Mannina L. 2009. NMR metabolic profiling of transgenic maize with the *Cry1A(b)* gene. *J Agric Food Chem* **57**: 6041–6049.
- Reeves RG. 1950. Morphology of the ear and tassel of maize. *Am J Bot* **37**: 697–704.
- Sakakibara H, Kawabata S, Hase T, Sugiyama T. 1992. Differential effects of nitrate and light on the expression of glutamine synthetases and ferredoxin-dependent glutamate synthase in maize. *Plant Cell Physiol* **33**: 1193–1198.
- Sakurai N, Hayakawa T, Nakamura T, Yamaya T. 1996. Changes in the cellular localization of cytosolic glutamine synthetase protein in vascular bundles of rice leaves at various stages of development. *Planta* **200**: 306–311.
- Tabuchi M, Sugiyama K, Ishiyama K, Inoue E, Sato T, Takahashi H, Yamaya T. 2005. Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase1;1. *Plant J* **42**: 641–651.
- Teixeira J, Pereira S, Cánovas F, Salema R. 2005. Glutamine synthetase of potato (*Solanum tuberosum* L. cv. Desiree) plants: cell- and organ-specific expression and differential developmental regulation reveal specific roles in nitrogen assimilation and mobilization. *J Exp Bot* **56**: 663–671.
- Tobin AK, Yamaya T. 2001. Cellular compartmentation of ammonium assimilation in rice and barley. *J Exp Bot* **52**: 591–604.
- Verpoorte R, Choi YH, Kim HK. 2007. NMR-based metabolomics at work in phytochemistry. *Phytochem Rev* **6**: 3–14.
- Whetten R, Sederoff R. 1995. Lignin biosynthesis. *Plant Cell* **7**: 1001–1013.
- Wong HK, Chan HK, Coruzzi GM, Lam HM. 2004. Correlation of ASN2 gene expression with ammonium metabolism in Arabidopsis. *Plant Physiol* **134**: 332–338.