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Rafael A. Canas, Isabelle Quillere, Peter J. Lea, Bertrand Hirel. Analysis of amino acid metabolism in the ear of maize mutants deficient in two cytosolic glutamine synthetase isoenzymes highlights the importance of asparagine for nitrogen translocation within sink organs. *Plant Biotechnology Journal*, 2010, 8 (9), pp.966-978. 10.1111/j.1467-7652.2010.00524.x . hal-01203928

**HAL Id: hal-01203928**

**<https://hal.science/hal-01203928>**

Submitted on 30 May 2020

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# Analysis of amino acid metabolism in the ear of maize mutants deficient in two cytosolic glutamine synthetase isoenzymes highlights the importance of asparagine for nitrogen translocation within sink organs

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Received 8 January 2010;

revised 3 March 2010;

accepted 11 March 2010.

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## Summary

Nitrogen (N) metabolism was characterized in the developing ear of glutamine synthetase deficient mutants (*gln1-3*, *gln1-4* and *gln1-3/gln1-4*) of maize exhibiting a reduction in kernel yield. During the grain-filling period, the metabolite contents, enzyme activities and steady-state levels of transcripts for marker genes of amino acid synthesis and interconversion were monitored in the cob and kernels. The ear of *gln1-3* and *gln1-3/gln1-4* had a higher free amino acid content and a lower C/N ratio, when compared to the wild type. The free ammonium concentrations were also much higher in *gln1-3/gln1-4*, and Asn accumulation was higher in *gln1-3* and *gln1-3/gln1-4*. The level of transcripts of *ZmAS3* and *ZmAS4*, two genes encoding asparagine synthetase, increased in the 'aborted kernels' of *gln1-3* and *gln1-3/gln1-4*. The results show that N metabolism is clearly different in developing and 'aborted kernels'. The data support the hypothesis that N accumulated in 'aborted kernels' is remobilized *via* the cob to developing kernels using Asn as a transport molecule. The two genes *ZmAS3* and *ZmAS4* are likely to play an important role during this process.

**Keywords:** amino acids, nitrogen, mutants, glutamine synthetase, remobilization, *Zea mays*.

## Introduction

Maize (*Zea mays* L.) is one of the most important crops grown worldwide for both human food and animal feed (Hirel *et al.*, 2007b). Maize exhibits C<sub>4</sub> metabolism, and therefore the concentration of atmospheric CO<sub>2</sub> does not limit the rate of photosynthesis. However, a reduction in the availability of N or water can cause a yield penalty (Cazetta *et al.*, 1999; Andrade *et al.*, 2002; Martin *et al.*, 2006). Therefore, over the last two decades, agronomic, physiological, molecular and quantitative genetic studies have been undertaken to improve our understanding of nitrogen (N) use in maize to increase its utilization efficiency (NUE), whilst at the same time maintaining an acceptable yield (kernel number and kernel size), especially under N-limiting conditions (Cazetta *et al.*, 1999; Gallais and Hirel, 2004; Hirel *et al.*, 2007a). For most crop species,

the plant life cycle can be roughly divided into two main phases: (i) the vegetative growth phase, when young developing roots and leaves behave as sink organs that efficiently take up and assimilate inorganic nitrogen (mainly in the form of nitrate and to a lesser extent ammonium) for amino acid and protein synthesis and (ii) the remobilization phase when senescing tissues start to behave as source organs, translocating organic molecules to ensure the formation of new developing and/or storage organs such as the ear in maize (Masclaux *et al.*, 2001). Therefore, during these two phases of plant growth and development, a better understanding of the metabolic and genetic control of nitrogen assimilation and recycling in the different organs of a plant is required.

Among the various biological processes involved in N assimilation and use, it has been recently shown that the synthesis and interconversion of amino acids is of major

importance during the development of all seeds and in particular in the maize ear (Seebauer *et al.*, 2004; Cañas *et al.*, 2009). The maize ear is composed of three main parts: (i) the cob, which is a complex of highly vascularized cellular structures that ensure the translocation of carbon and nitrogen assimilates from the shoots to developing kernels (Laubengayer, 1949); (ii) the 'aborted kernels', which are senescent pollinated or unpollinated ovules that do not increase in size in comparison with the developing kernels (Brink, 1927) and (iii) the developing kernels, which represents the ultimate storage organs for both carbon (C) and N that are not only used for human food and animal feed but also ensure plant survival for the next generation (Masclaux *et al.*, 2001). The efficient translocation of N assimilates to the ovules during the period of silking is vital to ensure optimal yield with respect to kernel set (Cazetta *et al.*, 1999). Therefore, a better understanding of the physiological and molecular processes occurring in the ear that are involved in the control of kernel set and kernel filling is of major importance for future biotechnological applications to improve plant productivity and for the rationalization of crop fertilization. In particular, identifying the key genes and metabolic pathways involved will help geneticists and breeders to select new traits related to NUE in relation to yield, taking into account plant functioning at the spatial (cellular and organ distribution) and temporal (changes in the physiology of the crop during growth and development) levels (Hirel and Lemaire, 2006; Hirel *et al.*, 2007a). However, studying such complex metabolic processes is not simple, as it is difficult to alter specifically N translocation from the leaves to the developing ear, without causing a major disturbance in the physiology of the plant. Even the use of  $^{15}\text{N}$ - or  $^{13}\text{C}$ -labelling experiments is not trivial when dealing with a complex structure such as the maize ear, characterized by the occurrence of different tissues and cell types. In addition, N translocation coming from the leaves and roots and N recycling within the ear appear to occur simultaneously (Seebauer *et al.*, 2004). Experiments in which labelled substrates were fed directly to detached developing kernels have been carried out previously (Muhitch, 1993; Glawischnig *et al.*, 2001). Although very informative with respect to the identification of possible pathways of amino acid metabolism, this type of experiment was only feasible using one organ that was separated from the plant and thus was only able to follow the unidirectional flux of reduced N *in vitro*.

In one of our previous studies, we have shown that mutants deficient in the two GS1 isoenzymes (GS1-3 and

GS1-4) are very useful material for deciphering amino acid metabolism in maize and its role on grain development and yield. These mutants exhibit perturbations in both the synthesis and translocation of N transport molecules in the shoots, thus leading to a reduction in grain yield without a noticeable modification of shoot growth and development (Martin *et al.*, 2006). Using a metabolic and gene expression approach, we have thus studied the impact of the two mutations on amino acid metabolism in three parts of the ear represented by the 'aborted' and developing kernels and the cob. Moreover, because the phenotype of the *gln1-3* mutant was characterized by a reduction in kernel number, whereas in the *gln1-4* mutant, kernel size was reduced (the effect of the two mutation being cumulative), it was interesting to investigate whether the perturbations caused by the *gln1-3*, *gln1-4* single mutations and *gln1-3/gln1-4* double mutation also affected amino acid metabolism in the developing ear.

Moreover, in a recent study it has been suggested GS1-3 and GS1-4 isoforms are likely to play an important role in the N economy of the plant, not only in the vegetative tissues but also in the reproductive organs during their development (Cañas *et al.*, 2009). In the ear, GS1 is thought to be involved in the production of Gln for transport and reallocation, to allow the synthesis of other amino acids such as Asn during the kernel-filling period (Seebauer *et al.*, 2004; Cañas *et al.*, 2009), which are further used for the synthesis of storage proteins.

We therefore developed an integrated approach to study the various events related to amino acid biosynthesis and interconversion during the development of the ear both in the wild type (WT) and in the three types of GS-deficient mutants. Because both during the period around silking and during grain filling, N availability is critical for translocation of C assimilates (Andersen *et al.*, 2002), we have determined the changes in a number of markers representative of C and N translocation and storage. To achieve this, we have examined the changes in the concentration of N and C metabolites, the activity of the main enzymes involved in ammonia assimilation and the abundance of transcripts encoding genes for the main enzymes involved in amino acid biosynthesis and interconversion.

This study allowed not only a further investigation into the role of *ZmGln1-3* and *ZmGln1-4* during ear development, but also the construction of a comprehensive picture of the various events related to N assimilation and recycling in the cob and kernels during the development of the ear. In particular, we have showed that the

phenotypes of the GS1 mutants could be predicted in the early phases of ear development, which roughly corresponds to the period around pollination. It is also likely that the ears of the mutants recycle the N accumulated in the 'aborted kernels' to allow the development of normal kernels. This particular process fits in well with the current consensus that in maize, Asn is a mobile intermediary N-containing molecule that can be quickly translocated to any part of the plant under certain physiological conditions, including a shortage of inorganic N.

## Results

### Metabolite and enzyme activity profiles

Metabolite and enzyme activity profiling in the 'aborted' and developing kernels and cob of WT and *gln1-3*, *gln1-4* and *gln1-3/gln1-4* mutant plants are presented in Figure 1. These metabolites and enzyme activities correspond to physiological markers representative of both the N and C status at a given stage of plant development both in the leaves (Hirel *et al.*, 2005a,b) and in the ears (Cañas *et al.*, 2009). These physiological markers include enzyme activities such as GS and glutamate dehydrogenase (GDH), metabolites such as ammonium, free amino acids, soluble sugars, starch, as well as total C and N and their ratio.

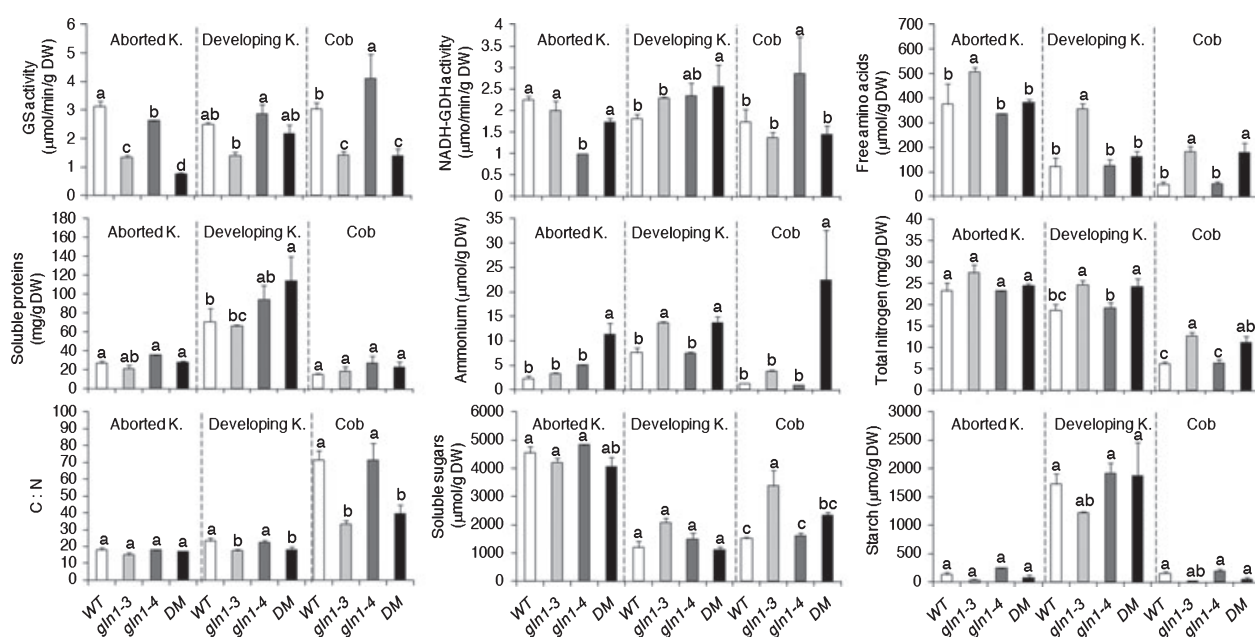
In general, in the three parts of the ear considered in this study ('aborted kernels', developing kernels and cob), the total GS activity determined in the *gln1-3* and *gln1-3/gln1-4* mutants was approximately 50% of that in the WT and *gln1-4* mutant.

The activity of NADH-GDH was similar in the majority of the analysed samples. It was only in the *gln1-4* mutant that the level of measurable enzyme activity was significantly lower in 'aborted kernels' and higher in the cob when compared to the WT ( $P \leq 0.05$ ).

In both the 'aborted' and developing kernels, the free amino acid content of the *gln1-3* mutant was significantly higher compared to the other mutants and WT. There were also higher amounts of free amino acids in the cob of the *gln1-3* and *gln1-3/gln1-4* mutants compared to the WT and the *gln1-4* mutant.

The soluble protein content of the developing kernels was considerably higher than the 'aborted kernels' and the cob. In the developing kernels, there was a significant increase in protein in the *gln1-3/gln1-4* mutant in comparison with the WT.

The free ammonium content of the 'aborted kernels' and cobs of the *gln1-3/gln1-4* double mutant was significantly higher, when compared to the other two mutants and WT. The free ammonium content was also significantly higher in the developing kernels of the *gln1-3* and



**Figure 1** Metabolite and enzyme activity profiles in the 'aborted', developing kernels and cob of wild type (white bars), *gln1-3* (clear grey bars), *gln1-4* (dark grey bars) and *gln1-3/gln1-4* (DM) (black bars) mutants. Samples were harvested at 25 DAS. Values are mean  $\pm$  S.E. of three independent plant samples. Different letters indicate significant differences between samples from the same organ ('aborted' kernel, developing kernel and cob) at  $P \leq 0.05$ .

*gln1-3/gln1-4* mutants, in comparison with that measured in the WT and *gln1-4* mutant.

Although low, a significant increase in the total N content was observed in the developing kernels and in the cob of the *gln1-3* and *gln1-3/gln1-4* mutants, when compared to that measured in the WT and the *gln1-4* mutant. Consequently, the C/N ratio was significantly decreased in both the developing kernels and cobs of the *gln1-3* and *gln1-3/gln1-4* mutants.

The soluble sugar content of all the tested samples was not markedly modified by genotype, except in the cob, where the soluble sugar content of the *gln1-3* mutant was significantly higher. Although the starch content was dramatically higher in the developing kernels, there were no major differences between the WT and the mutant samples.

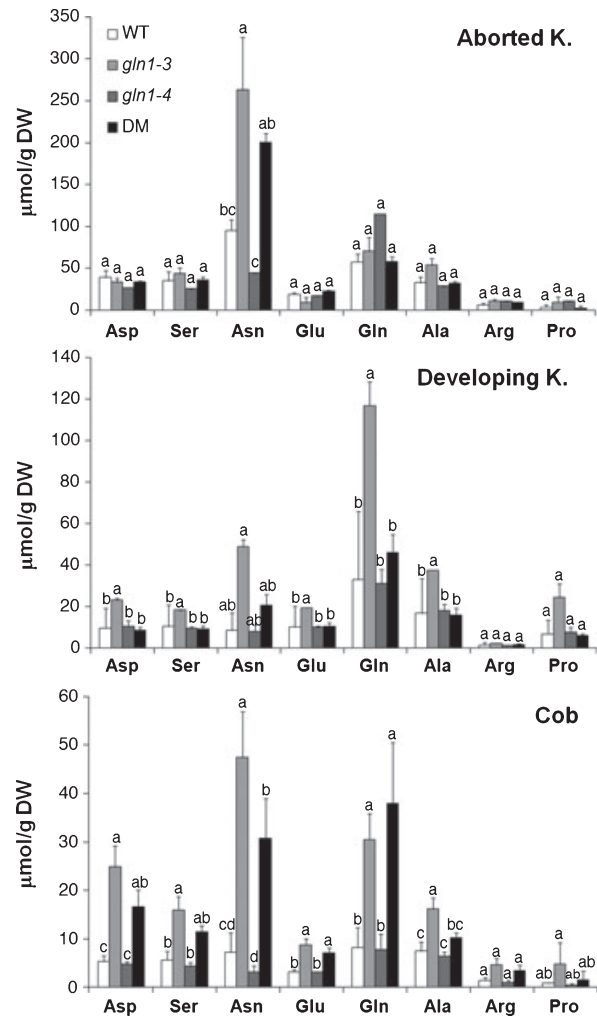
### Free amino acid profiles

The free amino acid profiles of the 'aborted kernels', developing kernels and cobs from the WT and the three mutant plants are presented in Figure 2. In the WT maize line B73, Asp, Ser, Asn, Glu, Gln, Ala, Arg and Pro represent from 65% to 84% of the total soluble amino acid pool in the three parts of the ear. Overall, Gln was the most abundant amino acid (11%–34%), whichever type of tissue examined. However, exceptions to this general trend were observed, because Asn predominated in the 'aborted kernels' of WT plants (Asn 24%; Gln 15%), *gln1-3* (Asn 44%; Gln 12%) and *gln1-3/gln1-4*-mutant plants (Asn 39%; Gln 11%). This was also the case in the cob of the *gln1-3* mutant (Asn 26%; Gln 16%).

In the 'aborted kernels', individual amino acids were present in similar concentrations in the different genotypes except for Asn, which was considerably increased in the *gln1-3* and in *gln1-3/gln1-4* mutants, when compared to either the WT or the *gln1-4* mutant. In the developing kernels of the *gln1-3* mutant, all amino acids were more abundant in comparison with the other genotypes, the Asp, Ser, Asn, Glu, Gln and Ala content being significantly higher. In the cobs of the *gln1-3* and *gln1-3/gln1-4* mutants, the concentration of Asp, Ser, Asn, Glu, Gln and Ala was significantly higher in comparison with the WT and *gln1-4*-mutant plants.

### Steady-state level of gene expression

Accumulation of mRNA of a number of genes encoding enzymes involved in amino acid (Asn, Asp, Ala, Glu, Gln

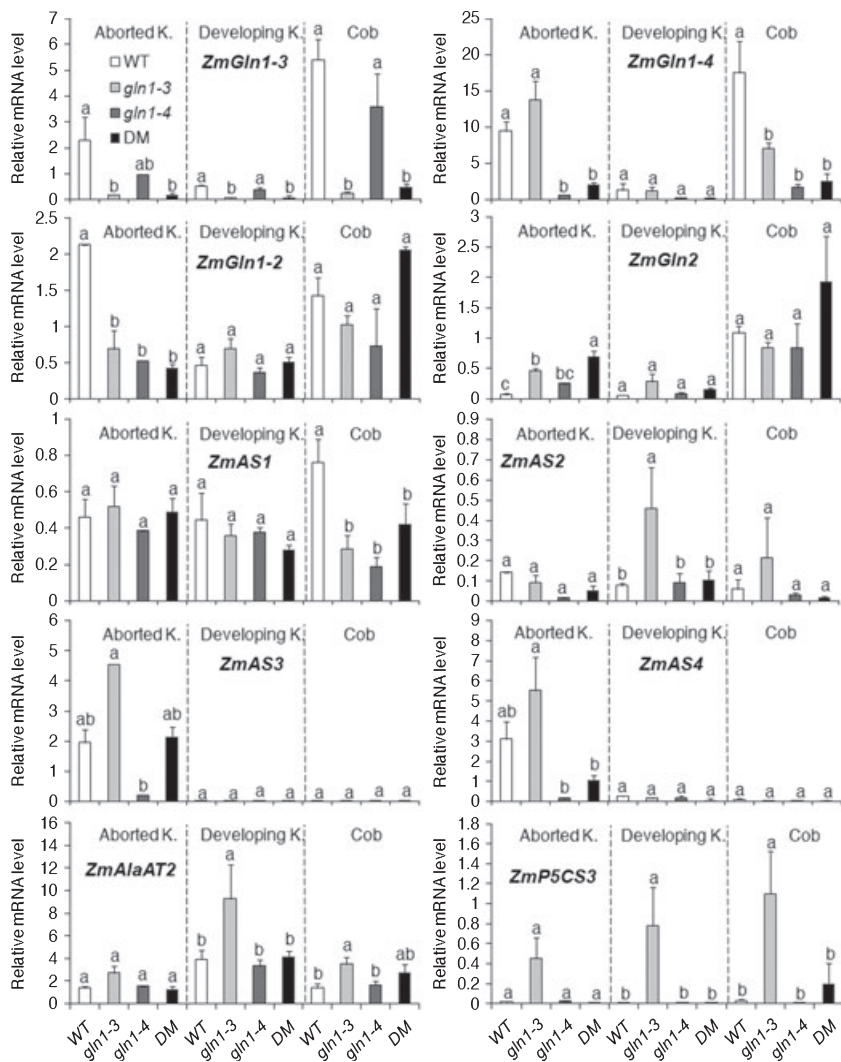


**Figure 2** Free amino acid profiles of the 'aborted' and developing kernels and cob of wild type (white bars), *gln1-3* (clear grey bars), *gln1-4* (dark grey bars) and *gln1-3/gln1-4* (DM) (black bars) mutants. Samples were harvested at 25 DAS. Values are mean  $\pm$  SE of three independent plant samples. Different letters indicate significant differences between samples from the same organ ('aborted' kernel, developing kernel and cob) at  $P \leq 0.05$ .

and Pro) biosynthesis and interconversion were quantified in the 'aborted kernels', the developing kernels and the cob of the WT and the three GS mutant plants. The results are presented in Figure 3.

The transcript abundance of *ZmGln1-3* (one of the five genes encoding cytosolic GS) was, as expected, very low in all parts of the ear, in both the *gln1-3* and the *gln1-3/gln1-4* mutants. In both the WT and the *gln1-4* mutant plants, the amount of *ZmGln1-3* transcripts was much higher, mainly in the cobs.

The transcript abundance of *ZmGln1-4* was very low compared to the WT in both the 'aborted kernels' and cobs of the *gln1-4* and *gln1-3/gln1-4* mutants and close



**Figure 3** Steady-state level of transcripts for enzymes involved in amino acid biosynthesis and inter-conversion in the maize (*Zea mays*) ear. Quantification was performed using qRT-PCR using total RNA samples extracted from 'aborted kernels', developing kernels and cob of wild type (white bars), *gln1-3* (clear grey bars), *gln1-4* (dark grey bars) and *gln1-3/gln1-4* (DM) (black bars) mutants. Samples were harvested at 25 DAS. Each value was normalized with the relative level of transcripts of the *EF1 $\alpha$*  gene. Values are mean  $\pm$  SE of three independent plant samples. Different letters indicate significant differences between samples from the same organ ('aborted' kernel, developing kernel and cob) at  $P \leq 0.05$ . *AlaAT*, alanine aminotransferase; *AS*, asparagine synthetase; *Gln*, glutamine synthetase; *P5CS*,  $\Delta^1$ -pyrroline-5-carboxylate synthetase.

to the background level in the developing kernels. In the 'aborted kernels' of the WT and in the *gln1-3* mutant, the level of *ZmGln1-4* transcripts was high and similar to that found in the cob. In the developing kernels, the level of transcripts of *ZmGln1-4* was strongly reduced compared to the other two parts of the ear. In the cob, the transcript abundance of *ZmGln1-4* in all the mutants was significantly decreased, in comparison with WT plants.

The abundance of *ZmGln1-2* mRNA was significant lower in the 'aborted kernels' of the three mutants compared to the WT. However, in both the developing kernels and the cob, the level of *ZmGln1-2* transcripts was not significantly modified either in the WT or in the three GS1 mutants.

For *ZmGln2*, the gene encoding plastidic GS (GS2), there were no major changes in the abundance of the corresponding transcripts, except in the 'aborted kernels' of the *gln1-3* and *gln1-3/gln1-4* mutants, in which the

mRNA levels were significantly increased. A slight increase in *ZmGln2* transcript level was also observed in the cobs of *gln1-3/gln1-4* mutants. Interestingly, this *ZmGln2* GS gene was expressed at a higher level in the cob, when compared to the two other parts of the ear.

The abundance of *ZmAS1* mRNA encoding asparagine synthetase (AS) was very similar in the 'aborted' and developing kernels of the WT and the three GS mutants, whilst abundance was significantly decreased in the cob of the three GS mutants. *ZmAS2* mRNAs were more abundant in the developing kernels and cobs of the *gln1-3* mutant, when compared to the other genotypes. However, it was only in the developing kernels that the observed differences were statistically significant. A considerable accumulation of *ZmAS3* and *ZmAS4* mRNAs was observed in the 'aborted kernels' of the WT, the *gln1-3* and the *gln1-3/gln1-4*-mutant plants but not in *gln1-4* mutant. Transcript abundance of these two genes was

close to zero, in both the developing kernels and cobs, irrespective of the genotype.

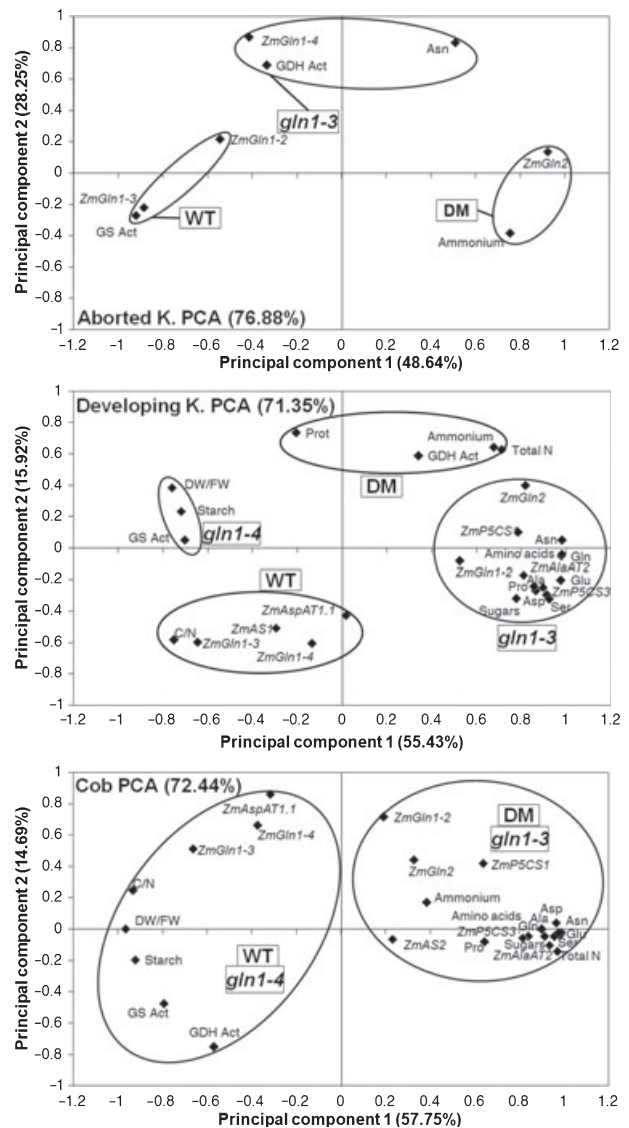
The abundance of mRNA for glutamate dehydrogenase (NADH-GDH: *ZmGDH1* and *ZmGDH2*) and aspartate aminotransferase (AspAT: *ZmAspAT1.1*, *ZmAspAT1.2*, *ZmAspAT1.3* and *ZmAspAT2.1*) was not significantly modified in the different parts of the ear of the three GS mutants in comparison with the WT (data not shown).

The abundance of *ZmAlaAT2* transcripts encoding alanine aminotransferase was higher in the developing kernels and cobs of the *gln1-3* mutant, when compared to that detected in the other genotypes. This strong up-regulation was also found for *ZmP5CS3* mRNA, which encodes  $\Delta^1$ -pyrroline-5-carboxylate synthetase, but this time in the 'aborted' and developing kernels and cobs. The transcript abundance of the *ZmP5CS1* and *ZmP5CS2* genes was not significantly modified in any of the parts of the ear, when the WT was compared to the three GS mutants (data not shown).

#### Metabolic and transcript abundance data integration

Principal component analysis (PCA) was carried out to visualize any correlative relationships that may exist between the steady-state levels of gene expression, enzyme activities and metabolite contents in the three parts of the developing ear of the WT and the three GS mutants. Using an ANOVA model type III, traits were selected with a *P* variance value  $\leq 0.05$ . Because the 'aborted kernels', the developing kernels and the cob are tissues in which different metabolic processes may occur, data analysis was performed on each individual part of the ear.

For the entire data set, the percentage of variance obtained between the WT and the three mutants was 76.88% for the 'aborted kernels', 85.37% for the developing kernels and 87.20% for the cobs. A first PCA analysis was performed for the different variables (transcript abundance, enzyme activities and metabolite contents) using the WT and the three GS mutants as a reference. In the graph visualizing the result of this type of clustering analysis, the impact of the three GS mutations on the repartition of the different variables can be seen (barycentre of a cluster of variables found for each of the four plant samples represented by the WT and three GS mutants) (Figure S1). Another type of PCA representation (Figure 4) shows the repartition of clusters of variables to determine whether the WT and the three GS mutants could be differentiated by specific relationships existing



**Figure 4** Principal component analysis of the different physiological and molecular traits related to N and C metabolism in the developing ear of maize (*Zea mays*). In the 'aborted kernels' (Aborted K.), developing kernels (Developing K.) and cob (Cob) of plants harvested 25 DAS. Values of the different traits were projected into three biplots of principal components arranged in descending order of variance. Each component allowed the identification of groups of metabolites, enzyme activities and levels of transcripts depending on the genotype: wild type, *gln1-3*, *gln1-4* and *gln1-3/gln1-4* (DM).

between these variables. In the 'aborted' and developing kernels, the traits were grouped into three clusters corresponding to the WT, *gln1-3*, *gln1-4* and *gln1-3/gln1-4* mutants. However in the 'aborted kernels', no particular cluster of variables was distinguishable for the *gln1-4* mutant. In the developing kernels, a cluster of variables grouping together the WT and *gln1-4* mutant and two groups of variables differentiating the *gln1-3* mutant and *gln1-3/gln1-4* double mutant were identified.

In the cob, PCA revealed the presence of two different clusters, with a different number of traits in each cluster. One of the clusters corresponded to traits that predominated in the WT and *gln1-4*-mutant plants, and the other was representative of the *gln1-3* mutant and the *gln1-3/gln1-4* double mutant. However, within each of the two clusters, the relationships existing between traits could be differentiated (tips of the double arrows).

## Discussion

### Impact of the GS mutations on physiological traits

A decrease in total GS activity was observed in all parts of the ear in both the *gln1-3* and *gln1-3/gln1-4* mutants (Figure 1). This decrease was similar in the single and double GS mutants, thus indicating that the loss of activity of the GS1-3 isoform is mainly responsible for the reduction in total GS activity. This finding confirms previous conclusions that transcript accumulation of *ZmGln1-3* is constitutive in all the maize organs examined so far (Limami *et al.*, 2002; Hirel *et al.*, 2005a; Cañas *et al.*, 2009). As *ZmGln1-3*, along with *ZmGln1-4*, are the most abundantly expressed GS1 genes in the ear, it is likely that these genes play an important role in the control of kernel set not only in vegetative organs but also in the reproductive parts of the plants. Interestingly, the finding that total GS activity was slightly increased both in the cob and in developing kernels of the *gln1-4* mutants suggests that unlike in the leaves (Martin *et al.*, 2006), there is a regulatory control mechanism up-regulating total GS enzyme activity in the cob that is specifically triggered when *ZmGln1-4* is impaired. However, the other genes coding GS were not up-regulated at the transcriptional level (Figure 3).

In the *gln1-3/gln1-4* mutant, the remaining enzyme activity probably results from the transcript accumulation of the two other genes encoding GS1 and GS2 (*ZmGln1-2* and *ZmGln2*), which would allow ammonium assimilation to ensure both plant survival and a vegetative development similar to that of WT plants (Martin *et al.*, 2006).

The accumulation patterns of the N metabolism markers such as free amino acids, free ammonium, soluble protein and total N (Figure 1) were rather complex in the three mutants, because specific differences were observed either linked to the mutation, to the part of the ear examined or to the interaction of both parameters.

Compared to the WT and the single GS mutants, the level of free ammonium in the cob of the *gln1-3/gln1-4* double mutant was strongly enhanced (Figure 1). Such an

accumulation was previously observed in the leaves of the GS double mutant (Martin *et al.*, 2006), thus confirming that GS1-3 and GS1-4 act in concert to assimilate or re-assimilate the ammonium released in the organs and cell compartments in which N remobilization and N translocation is occurring.

Detailed analysis of the amino acid composition provided additional information on the complex regulation of N metabolism and N translocation occurring in the three parts of the developing ear (Figure 2). In the *gln1-3* and *gln1-3/gln1-4* mutants, an accumulation of most of the major amino acids in the cob suggests that the few remaining kernels that develop normally do not have the capacity to utilize the entire remobilized N for storage protein synthesis. Consequently, the supply of nutrients from the sink organs exceeds the assimilation capacity of the remaining developing kernels, thus provoking an important accumulation of Gln in the cobs of the *gln1-3* and *gln1-3/gln1-4* mutants exhibiting reduced GS activity. This finding indicates that the lack of GS1-3 isoenzyme causes strong perturbations in the use of N for kernel set (Martin *et al.*, 2006), which later on induces an accumulation of remobilized N as a result of sink strength limitation. Because in the *gln1-4* mutant, the kernel number was not greatly reduced (Martin *et al.*, 2006), it was logical to find that such an accumulation of amino acids did not occur in any parts of the ear.

In developing kernels, the pattern of individual amino acid accumulation (Figure 2) was different, because in the *gln1-3* mutant an increase in all the major amino acids was observed, Gln being the most abundant. This could explain why lower amounts of soluble proteins were detected in the kernels of the *gln1-3* mutant, as the amino acids were not used for protein synthesis. The lack of an increased concentration of amino acids, in particular Gln and Asn, in the *gln1-3/gln1-4* double mutant, is more difficult to explain. It is possible that there is a non-additive effect of the two mutations with respect to amino acid translocation to the kernels and their use for protein synthesis or there is a counteracting effect of the *gln1-4* mutation.

In the 'aborted kernels', the situation was again different, because only an accumulation of Asn was observed in the *gln1-3* and *gln1-3/gln1-4* mutants (Figure 2). Seebauer *et al.* (2004) observed that in unpollinated ovules, there was a large increase in the concentration of Asn in comparison with that detected in developing kernels. This accumulation of Asn could have originated either from N recycling in the 'aborted kernels' or from N remobilization



from the shoots and transport *via* the cob. However, Gln, the other major amino acid used for N translocation from the cob, did not accumulate in the 'aborted kernels', which is in favour of the first hypothesis. It is possible that ammonia was incorporated directly into asparagine without passing through glutamine. Ammonia is able to act as a substrate for maize AS *in vitro* (Oaks and Ross, 1984), although all of the plant AS proteins sequenced so far have a glutamine-binding site (Todd *et al.*, 2008). Tobacco leaves fed with azaserine, an inhibitor of the glutamine-dependent AS reaction, were able to incorporate <sup>15</sup>N-labelled ammonia into asparagine, indicating that the reaction could occur *in vivo* under certain circumstances (Masclaux-Daubresse *et al.*, 2006). In the cobs of *gln1-3* and *gln1-3/gln1-4* mutants, the total N contents were higher, which led to lower C/N ratios, when compared to the WT and *gln1-4* mutant. This observation further supports the current conclusion that N assimilation and N recycling and thus N availability is the major limiting factor controlling kernel set (Below, 1997; Martin *et al.*, 2006).

The differences observed in amino acid content highlight the complex pattern of metabolic regulation of N metabolism occurring in the three parts of the ear, which apparently results from a specific interaction occurring between a given mutation (*gln1-3*; *gln1-4* or both mutations) and the type of tissue examined. In contrast, no major differences were observed between the WT and the different mutants with respect to the soluble and insoluble carbohydrate contents, which were not significantly modified in any of the parts of the ear (Figure 1). Thus, the main conclusion of this part of the study is that the two GS mutations influence N metabolism in the ear and not only in the leaf and root (Martin *et al.*, 2006).

### Impact of the GS mutations on transcript abundance and its physiological meaning

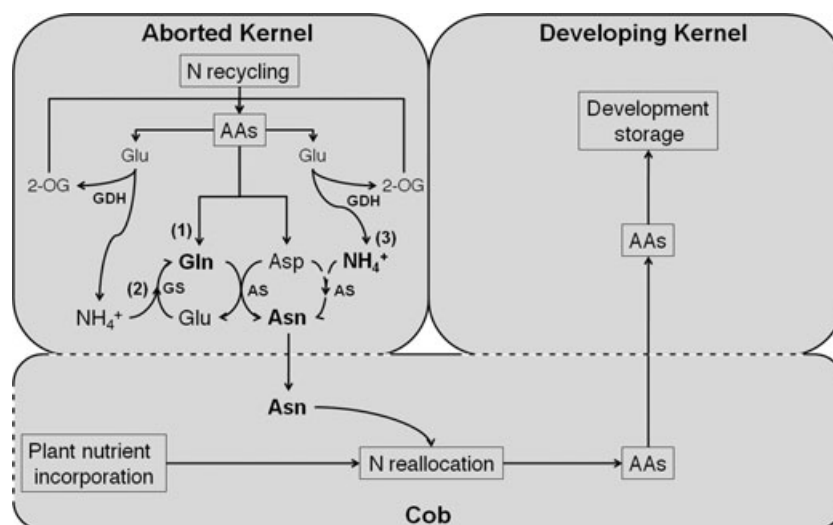
We have confirmed that in the WT maize line B73, there is a high level of transcripts of the *ZmGln1-3* and *ZmGln1-4* genes in the cob (Figure 3) as previously found in other maize lines (Cañas *et al.*, 2009). However, as seen in the leaves and roots of the *gln1-3* and *gln1-4* mutants (Martin *et al.*, 2006), transcripts of *ZmGln1-3* were at the limit of detection, and transcripts of *ZmGln1-4* were strongly reduced (Figure 3). Because total GS activity in the cob was significantly reduced only in the *gln1-3* mutants, this indicates that GS1-3 is the main active GS isoform in this organ. Interestingly, we also found that *ZmGln1-4* is expressed at a much higher level in 'aborted kernels' compared

to the developing ones, which is in line with the data obtained from the physiological studies, suggesting that N metabolism is different in the two types of organs.

The mutations had an impact on the accumulation of *ZmGln1-2* transcripts encoding a GS isoform located in the vasculature (Muhitch *et al.*, 2002; Martin *et al.*, 2006), only in 'aborted kernels' (Figure 3), which is probably linked to the perturbation of N assimilation in this organ. In contrast, a slight increase in the level of transcripts for plastidic *ZmGln2* in the 'aborted kernels' is probably compensating partly for the reduction in GS activity, at least in the *gln1-3* mutants, as previously observed in the leaves (Martin *et al.*, 2006).

Other results from mRNA accumulation studies confirmed that N metabolism is strongly modified in the ear of the GS mutants (Figure 3). These modifications were detected mainly in the 'aborted kernels' and to a lesser extent in the two other parts of the ear. The changes in transcript abundance were mainly related to Asn metabolism in line with the amino acid profiling study. In the 'aborted kernels', the transcript accumulation of the AS genes, *ZmAS3* and *ZmAS4*, was enhanced in the WT and in the in the two mutants harbouring the *gln1-3* mutation, whilst transcript abundance was lower in the *gln1-4* mutant (Figure 3). Thus in 'aborted kernels', a reduction in the transcript abundance of both AS genes is linked to the impairment of expression of *ZmGln1-4*. It is also worth stressing that the transcript abundance of *ZmAS4* was at the limit of detection in most maize organs or tissues examined so far (Cañas *et al.*, 2009), thus highlighting a specific function in 'aborted kernels'. The finding that a high level of *ZmAS4* mRNA accumulation was also found in senescing leaves further suggests a role for AS4 during N remobilization (data not shown).

The data support the hypothesis that the changes in *ZmAS3* and *ZmAS4* transcript abundance, together with the changes in the Asn content in the GS mutants, are probably linked to the processes of N recycling and N remobilization of 'aborted kernels'. This suggests that N remobilization occurs in the 'aborted kernels', in which proteins are broken down and recycled, thus increasing the free amino acid content. These amino acids are recycled in the form of Asn, which appears to be the preferred transport amino acid between the 'aborted kernels' and the cob (Figure 5). The genes products of *ZmAS3* and *ZmAS4* are likely to be involved in the control of this amino acid conversion process. Our data also suggest that both the Gln released from protein hydrolysis and synthesized by the remaining GS activity may be sufficient to



**Figure 5** Schematic representation of a putative metabolic pathway for N assimilation and recycling in the developing ear of maize (*Zea mays*). In the 'aborted kernels', N is mainly recycled in the form of Asn which is translocated via the cob to developing kernels. Substrates for Asn synthesis may arise from (1) Gln released during protein hydrolysis; (2) *de novo* Gln synthesis and (3) free ammonium released by the GDH reaction. The GDH enzyme maintains the steady-state level of Glu during protein breakdown and N recycling (Forde and Lea, 2007; Labboun *et al.*, 2009). (AAs, amino acids; AS, asparagine synthetase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; 2-OG, 2-oxoglutarate).

feed the AS reaction. However, the possibility that the ammonium not assimilated by GS in the mutants is incorporated directly into Asn through the reaction catalysed by AS cannot be ruled out (Lea *et al.*, 2007). The finding that large amounts of *ZmGln1-4* mRNAs are detected in the 'aborted kernels' further supports this hypothesis, because we previously found that it is likely to be involved in the process of N remobilization in the leaf, whilst *ZmGln1-3* is required for N assimilation (Hirel *et al.*, 2005a; Martin *et al.*, 2005, 2006).

The data also show that the abundance of the transcripts for *ZmAS2*, *ZmAlaAT2* and *ZmP5CS3* was up-regulated in the developing kernels of the *gln1-3* mutants (Figure 3). Although at this stage of investigation, it is difficult to interpret such changes in patterns of transcript accumulation, they seem to be directly or indirectly linked to the *gln1-3* single mutation, which might explain why the phenotype of the developing ear is unique in this mutant. There are approximately twice as many, larger, kernels in *gln1-3*, in comparison with the *gln1-3/gln1-4* double mutant.

#### Metabolic and transcript abundance data integration to decipher the complex physiological impact of the GS mutations

PCA was carried out to obtain further information on the correlative relationships existing between three classes of

traits represented by transcript abundance, enzyme activity and metabolite content in the WT and the three GS mutants.

In 'aborted kernels', three different clusters of correlative traits are visible, corresponding to the WT, *gln1-3* and *gln1-3/gln1-4* mutants, whereas no correlations were found for the *gln1-4* mutant (Figure 4). This observation suggests that there are specific metabolic and transcript accumulation responses in the 'aborted kernels' in the WT and the two GS mutants. Although a limited number of correlative traits were found in the three clusters, it is worth noting that the genes *ZmGln1-2* and *ZmGln1-3* are located in different clusters from *ZmGln1-4* and *ZmGln2* (Figure 4). This could mean that there is a complex interaction between the expressions of these genes including compensatory mechanisms when one of them is down-regulated, to maintain a minimum level of GS activity. It is also worth noting that the cluster in the 'aborted kernels' of the *gln1-3* mutant contains the level of transcripts of *ZmGln1-4*, GDH activity and the Asn content, thus confirming that a process linked to N remobilization is specifically occurring in the 'aborted kernels'. GS activity is linked to accumulation of *ZmGln1-2* and *ZmGln1-3* transcripts in the WT (Figure 4), because both genes are controlling the enzyme activity in this organ. *ZmGln2* could be involved in the reassimilation of ammonium, which accumulates in the *gln1-3/gln1-4* double mutant.

In developing kernels, three main clusters of traits were identified (Figure 4). One grouped the WT and *gln1-4* mutant, the two others were represented by the *gln1-3* mutant and *gln1-3/gln1-4* mutants. This observation suggests that in the *gln1-4* mutant; the physiology of this organ is similar to that of the WT because the number of developing kernels is almost identical in both types of plants.

This result fits the hypothesis that *ZmGln1-3* is controlling kernel set and *ZmGln1-4* is involved in the control of grain filling at much later stages of kernel development (Martin *et al.*, 2006). In contrast, a large number of traits were linked to the *gln1-3* mutation which was not the case for the *gln1-4* mutation. In the double mutant, only two traits were clustered, this is probably attributed to some counteractive effect of the two mutations.

Two clusters of traits were found for the cob, one again grouping the *gln1-4* mutant and the WT together and the other the *gln1-3* mutant and *gln1-3/gln1-4* double mutant (Figure 4). These results confirm that in organs involved in the translocation or supply of reduced N (shoots, cobs), it is the GS1-3 isoform that is controlling these two processes. Moreover, it is clear that the global status of the ear with respect to the presence or absence of 'aborted kernels' has a strong influence on the process of N translocation occurring in the cob.

## Conclusion

The study provides new information about the function of two genes encoding cytosolic GS (*ZmGln1-3* and *ZmGln1-4*) and of other genes involved in amino acid biosynthesis and interconversion in the developing ear during the kernel-filling period. These two genes appear to be involved not only in the control of N metabolism in the shoots but also in the developing ear. It is probable that the N that is assimilated by the 'aborted kernels' is then recycled and transported to the developing kernels via the cob through the putative metabolic pathway illustrated in Figure 5. This pathway also implies that there is a flux of reduced N occurring between the 'aborted kernels' via the cob to the developing kernels for optimal N utilization.

## Experimental procedures

### Plant material and growth conditions

Maize (*Z. mays* L., taxonomy ID: 4577; B73 line) WT and *gln1-3*, *gln1-4* and *gln1-3/gln1-4* mutants were obtained by *Mu* insertion events as described previously in detail by Martin *et al.* (2006).

Plants were grown in the field from May to September 2006 and 2008 at the Institut National de la Recherche Agronomique, Versailles, France. Seeds were first sown on coarse sand, and after 1 week, when two to three leaves had emerged, plantlets were transplanted to the field. The residual N provided by the soil was estimated at approximately 60 kg/ha. Both phosphorus (P205) and potassium (K20) were also applied at 100 kg/ha. Plant density was 99,000 plants/ha with a space of 80 cm between rows and approximately 16 cm between plants. The mutants were grown side by side in separate lines of 25 plants with an outside boarder area of 3 m on each side of a line of mutant plants. Three plants for the WT and each of the three mutants were harvested per line for the physiological and molecular studies. Ears of WT and mutant plants were harvested at 7, 14 and 25 days after silking (DAS). In the maize line F2, these harvesting times corresponded to the lag phase, the beginning of starch accumulation and the maximum starch accumulation rate, respectively (Prioul *et al.*, 2008). In the present investigation, line B73 was used as a genetic background for selecting the GS mutants. In this line, the most significant changes in the metabolic profiling were observed 25 DAS (Figure S2). Therefore, 25 DAS was chosen for further biochemical and molecular studies, because significant differences were observed in the grain-filling pattern, depending on the genetic background (Borrás *et al.*, 2009). Similar results were obtained over the 2 years 2008 and 2006, thus the detailed biochemical and molecular analyses of the ear samples were performed with those harvested in 2008. After harvesting of the ears, husks and shanks were discarded, and the remaining plant material was separated into cobs and developing kernels (including pedicels). In the ears harvested at 25 DAS, 'aborted' and developing kernels were easily separated as they exhibited a clearly different phenotype (Figure S3). All the samples were immediately frozen in liquid N. All frozen tissues were reduced to a homogenous powder using a grinder (MM301, Retsch, Haan, Germany). The powder was stored at  $-80^{\circ}\text{C}$  until extraction. Part of the powder was lyophilized using a freeze dryer (Gamma 1-16 LSC, Christ, Osterode am Harz, Germany). All the harvesting of fresh material was carried out concomitantly between 10 a.m. and 1 p.m.

### Metabolite and enzyme activity measurements

The soluble protein content of extracts was determined using a commercially available kit (Coomassie Blue protein assay reagent, Biorad, München, Germany) using bovine serum albumin as a standard (Bradford, 1976). Enzymes were extracted from frozen material stored at  $-80^{\circ}\text{C}$ . All extractions were performed at  $4^{\circ}\text{C}$ . GS transferase activity was measured according to the method of O'Neal and Joy (1973). GDH (NADH-GDH) was measured as described by Turano *et al.* (1996) except that the extraction buffer was the same as for GS (25 mM Tris pH7.6; 1 mM EDTA; 1 mM  $\text{MgCl}_2$ ; 10 mM  $\beta$ -mercaptoethanol; 40  $\mu\text{M}$  leupeptin).

$\text{NH}_4^+$  and amino acids were extracted from frozen samples with 2% 5-sulphosalicylic acid (100 mg FW/mL) (Ferrario-Méry *et al.*, 1998). Total free amino acids were determined by the Rosen colorimetric method using Gln as a standard (Rosen, 1957). The composition of individual amino acids, used to establish the free amino acid profiles, was determined by ion exchange chromatography followed by detection with ninhydrin using an AminoTac JLC-500/V amino acid analyzer according to the instructions of

the manufacturer (JEOL, Tokyo, Japan). Free  $NH_4^+$  was determined by the phenol hypochlorite assay (Berthelot reaction) (Husted *et al.*, 2000). The N and C contents of each fraction were determined by dry combustion in an autoanalyser (NC 2500; CE instruments, Wigan, UK).

Sucrose, glucose, fructose and starch were extracted from aliquots (10 mg DW) of lyophilized plant material using a three-step ethanol-water procedure as described by Lemaître *et al.* (2008). The soluble sugars (glucose, fructose and sucrose) were measured enzymatically using a commercially available kit assay (Roche, Boehringer Mannheim, Mannheim, Germany). Starch content was determined as described by Ferrario-Méry *et al.* (1998).

### RNA extraction, RT-transcription and qRT-PCR analysis

Total RNA was extracted as described by Verwoerd *et al.* (1989) from frozen plant material stored at  $-80^\circ\text{C}$ . Reverse transcription reactions and quantitative first strands were synthesized according to Daniel-Vedele and Caboche (1993) using M-MLV reverse transcriptase and oligo(dT) 15 primers (Invitrogen, Carlsbad, CA, USA). The PCR was performed on a Mastercycler® ep *realplex* instrument (Eppendorf, Hamburg, Germany) with the Mesa Fast qPCR Mastermix Plus for SYBR Assay (Eurogentec, Liege, Belgium) according to the manufacturer's protocol. Each reaction was performed on a 1 : 10 dilution of the first cDNA strand synthesized as described previously, in a total reaction of 20  $\mu\text{L}$ . With this dilution, the SYBR green signal was linear. Results were normalized by employing the transcript accumulation of the elongation factor 1 $\alpha$  gene (*EF1 $\alpha$* ; EU960821) as a constitutive reference using ( $2^{-\Delta\text{Ct}}$ )100. *EF1 $\alpha$*  was chosen as the reference gene from a set of 21 genes selected from a microarray experiment, which was further validated by qRT-PCRs using cDNAs from different organs and growth conditions. Each pair of primers was previously used in a RT-PCR to confirm the gene specificity amplification, and their products were sequenced. The reaction efficiency of primer pairs was also calculated using an amplification standard curve. The slope of the standard curve gave the efficiency of the PCR by the following equation: Efficiency =  $10^{(-1/\text{slope})} - 1$ .

### Primer design and sequences

The primers used for qRT-PCR were the same as those used in a previous study (Cañas *et al.*, 2009) except for the *ZmGln1-3*, *ZmAS2* and *ZmAlaAT2* genes. For *ZmGln1-3*, different primers were used because there is an allelic difference between the mutant and WT line B73 used in the present investigation and the two lines (F2 and lo) studied previously. For *ZmAS2* and *ZmAlaAT2*, new primers were used that exhibited better amplification efficiencies. The sequences of the primers used in qRT-PCR are presented in Table S1.

### Statistics

For metabolite analyses, enzyme activity measurements and quantification of mRNA abundance by qRT-PCR, the results are

presented as mean values for three individual plants with standard errors [SE =  $SD/\sqrt{(n-1)}$ ]. Statistical analyses were performed using the ANOVA and PCA function of the XLStat-Pro 7.5 (Addinsoft, New York, NY, USA) software. For clarity in the figures, only the results of three independent ANOVA analyses (for 'aborted kernels', developing kernels and cobs) are presented. Significant differences ( $P \leq 0.05$ ) are considered between the three mutants and WT plants.

### Acknowledgements

We are particularly grateful to Professor Keith Edwards of the University of Bristol for providing the GS mutants. The composition of individual amino acids and N and C content was carried out at the Plant Chemistry Platform of the Institute Jean-Pierre Bourgin, INRA, Versailles-Grignon. Rafael A. Cañas was supported by a post-doctoral fellowship from the Spanish Ministry of Science and FECYT.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Principal component analysis of the different physiological and molecular traits showing the position of the barycenters of the samples grouped for the wild type and for each glutamine synthetase mutant.

**Figure S2** Metabolite and enzyme activity profiles determined in 'aborted' kernels, developing kernels and cob of wild type, *gln1-3*, *gln1-4* and *gln1-3/gln1-4* (DM) mutants.

**Figure S3** Phenotype of the ear in the wild type and in the three maize glutamine synthetase mutants showing the proportion of 'aborted' and developing kernels.

**Table S1** Primers used in qRT-PCR.

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