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Over-expression of glutamine synthetase genes *Gln1-3/Gln1-4* improved nitrogen assimilation and maize yields

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Abstract

In agriculture, certain fertilizers that contain nitrogen generally tend to provide the most macronutrients for plant growth and development. The cDNAs of *Gln1-3* and *Gln1-4* genes, encoding glutamine synthetase isoenzymes (GS1), were fused to the rice actin1 promoter and over-expressed in the inbred maize line DH9632 by Agrobacterium-mediated genetic transformation. PCR assays demonstrated the integration of these genes in six transgenic lines. Transcription of *Gln1-3* or *Gln1-4* in the transformants was also confirmed by semi-quantitative RT-PCR and qRT-PCR; the transgenic lines had significantly higher expression compared with wild type. Transgenic lines L2 and L7 expressed the most Gln1-3 and Gln1-4 mRNA, respectively, and had the most enzyme activity in leaves below the ear after pollination for 14 days. Over-expression of these two genes led to increased chlorophyll content and improved photosynthesis after 14 days. In addition, yield-related traits such as ear length, ear diameter, ear weight, grain weight per ear, and hundred-kernel weight were improved in the transgenic lines. The plot yield of transgenic L2 was increased by approximately 20%. These results suggest that overexpression of Gln1-3 and Gln1-4 in maize improves yields and enhances nitrogen using efficiency. Thus, transgenic lines overexpressing Gln1-3 or Gln1-4 in maize could potentially be used in maize breeding.

Keywords: nitrogen, glutamine synthetase, transgene, yield, *Zea mays*

Abbreviations: GOGAT - glutamate synthase, GS - glutamine synthetase, RT-PCR - reverse transcription polymerase chain reaction, WT - wild-type, EPSPS - 5-enolpyruvyl-shikimate-3-phosphate synthase, qRT-PCR - quantitative real-time polymerase chain reaction

Introduction

Nitrogen fertilizer supplies one of the most macronutrients for plant growth and development and exists in the environment in several inorganic forms. Plants acquire nitrogen from nitrate or ammonium ions in soil. These compounds are converted to ammonia via sequential reduction by nitrate and nitrite reductases and, in legumes, through symbiotic fixation of atmospheric nitrogen (Lea and Ireland, 1999).

Many genes encoding enzymes involved in nitrogen metabolism in cereals have been successively identified and cloned using genetic engineering, for example, nitrate reductase, nitrite reductase, glutamate synthase (GOGAT), and glutamine synthetase (GS, EC 6.3.1.2) (Martin et al, 2006; Bernard et al, 2008; Tanaka et al, 2009). GS catalyses the ATP-dependent condensation of NH_3 with Glu to form Gln. GOGAT transfers the amino group of Gln to α-ketoglutarate to subsequently produce Glu (Temple et al, 1998; Ireland and Lea, 1999).

In cereals, GS may be a major check point controlling plant growth and productivity (Hirel et al, 2005a; Kichey et al, 2006). Two groups of GS isoenzymes, cytosolic (GS1) and plastidic (GS2), have been identified in higher plants by their subcellular localisation. GS1 proteins are part of a five-gene family, and GS2 is predominantly found in chlorophyllous tissues in higher plants (Kazunari et al, 1992). In higher plants, changes in plastidic (GS2) and cytosolic (GS1) GS isoenzyme concentrations indicate the transition of sink leaves to source leaves during leaf ageing (Masclaux et al, 2000; Terce-Laforgue et al, 2004). GS1 is involved in grain filling (Martin et al, 2006), proline accumulation in phloem (Brugière et al, 1999), amino acid interconversion during seed germination and glutamine synthesis for nitrogen transport during leaf aging. GS2 is involved in the fixation of ammonium during the photorespiratory nitrogen cycle (Akiko et al, 1996; Mitsuhiro et al, 2000; Giuseppe, 2000).

With the development of genetic engineering, many studies of GS genes have been performed in transgenic plants. Transgenic tobacco plants with the CaMV 35S promoter driving an alfalfa GS1 gene have increased GS activity in leaves (Temple et al, 1993). Tobacco plants over-expressing GS have increased growth (Andrea et al, 2000). When exposed to soy-

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bean GS1, the hairy roots of *Brassica napus* have more abundant GS polypeptide, approximately 3–6-fold greater GS activity and lower ammonia content (Downs et al, 1994).

Gln1-3 and *Gln1-4* are members of the GS1 gene family. *Gln1-3* and *Gln1-4* are highly expressed regardless of leafage or the level of N fertilization, although *Gln1-4* transcripts are increased in older leaves (Hirel et al, 2005a). *Gln1-4* encodes a GS isoform that re-assimilates ammonium released during leaf protein remobilization, whereas *Gln1-3* encodes a GS isoform that plays a housekeeping role and provides sufficient N assimilates to the developing ear to avoid kernel abortion (Hirel et al, 2005b). Martin et al (2006) reported that GS1-3 is present in mesophyll cells, whereas GS1-4 is specifically localized in the bundle sheath cells. Furthermore, the kernel number increased by 30% in a line over-expressing *Gln1-3*.

Globally, maize is one of the most important crops for both human food and animal feed production (http://apps.Fao.org). Maize productivity is strongly tied to two yield traits: kernel number and kernel weight (Cazetta et al, 1999; Gallais and Hirel, 2004; Martin et al, 2006). Nitrogen is the key input required for optimal maize production (Cazetta et al, 1999). To enhance maize yields by improving the efficiency of N use, genetic improvement and optimization of agronomic practices could be combined to alleviate food stress and decrease the excessive application of nitrogen fertilizer.

In this study, we cloned full-length of Gln1-3 and Gln1-4 cDNAs by RT-PCR and transferred them into maize (*Zea mays* L) by Agrobacterium-mediated transformation. Under normal and suboptimal nitrogen fertilizer conditions, the over-expressed plants of *Gln1-3* or *Gln1-4* have enhanced photosynthesis rates, chlorophyll contents, GS activity and yields compared with WT. This suggests that overexpression of Gln1-3 or Gln1-4 for cytosolic GS1 can enhance the efficiency of nitrogen use and could be used to improve kernel yields in breeding program in the future.

Materials and Methods

Cloning of Gln1-3 and Gln1-4 genes in maize

Maize cDNA was synthesised with a SMART™ cDNA Synthesis Kit (Sangon) using the manufacturer's instructions. Primers were designed from the maize Gln1-3 (GenBank Accession Number: X65928.1) and Gln1-4 (GenBank Accession Number: X65929.1) sequences. The primer sequences are shown in Table 1. PCR amplification was performed as follows: 95°C for 5 min; 95°C for 45 s, 54°C for 45 s, and 72°C for 2 min for 36 cycles; final extension at 72°C for 7 min. The resulting products were separated on a 1.0% agarose gel, extracted (Agarose Gel DNA Purification Kit, Xygen), cloned into the pGEM-T easy vector (Promega, USA), and sequenced by Bioasia, Inc (Shanghai, China).

Table 1 - Sequences of PCR primer pairs for target genes.

Name	Primer pairs
Gln1-3	P1: 5'-CAACCCCTGTCGCACCGCA-3'; P2: 5'- CACCACGACGACCTGACCCCG -3'
Gln1-4	P3: 5'-C CCCTCATTCCTCCTTGGG-3' P4: 5'- GGATGCAGCATGACACGTCTA -3'
PActin-Gln1-3	P5: 5'-CGCAATTCACCGATTATACATG-3' P6: 5'-CGCCGATTCCACAGTAGTAAG-3'
PActin-Gln1-4	P7: 5'-CCGATTATACATGCCAAGGT-3' P8: 5'-GCCGATTCCACAGTAGTAAG-3'
Epsp	P9: 5'-CCGCTCGA GATGAGCCCAGAACGACGCC -3' P10: 5'-CCGCTCGAGTCAGATCTCGGTGACGGGC -3'
QGln1-3	P11: 5'- GCCCAAGTGGAACTACGA-3' P12: 5'-TGTTGCCCCTCCTGAATG-3'
QGln1-4	P13: 5'- GAACTACGACGGCTCCA-3' P14: 5'- GTTGCCCCTCCTGAATG -3'
Actin	P15: 5'-ATCACCATTGGGTCAGAAAGG-3' P16: 5'-GTGCTGAGAGAAGCCAAAATAGAG-3'

Vector construction

The coding sequence of *Gln1-3* or *Gln1-4* was inserted into the *XbaI* and *KpnI* site of plasmid pCAMBIA1300-PActin::MCS-nos-epsp, which contains a rice constitutive *actin1* promoter (PActin), a nos terminator and the EPSPS gene (Figure 1). The resultant plasmid was transferred into *Agrobacterium tumefaciens* strain LBA4404 via the freeze-thaw method. Maize transformation was performed as described by He et al (2013). Herbicide-resistant transformed plants were selected and self-pollinated for two generations, and T2 plants were analyzed by PCR with primers for transgenes (Table 1). For PCR of transgenic plants, genomic DNA was isolated from maize leaves by the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook et al, 2000). Expression of the two genes in T2 transgenic plants was confirmed by semi-quantitative RT-PCR and qRT-PCR.

Overexpression of Gln1-3 or Gln1-4 in the transgenic lines

Transgenic lines and WT were assayed for Gln1-3 or Gln1-4 gene expression by RT-PCR and qRT-PCR and the former primers were the same as qRT-PCR. Total RNA was extracted from young leaves with Trizol reagent (Sangon) and treated with RNase-free DNase (Takara, Dalian, China). cDNAs were diluted 5-fold for PCR, according to the manufacturer's protocol. qPCR was performed in an optical 96-well plate using an ABI 7500 system (ABI, USA) with the SYBR GreenI RT-PCR Kit (Takara, Dalian, China) as described in He et al (2013). Primers of QGln1-3 or QGln1-4 (Table 1) were designed to amplify the transgenes, and actin was used as the endogenous control. QRT-PCR amplification conditions were as follows: 30 s at 95°C; 40 cycles of 10 s at 95°C, 34 s at 59°C. *Gln1-3* and *Gln1-4* transcript levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Enzyme extraction

Total GS activity was determined with the method described by O'Neal and Joy (1973) with some modifications. Approximately 1 g leaf (below the ear after

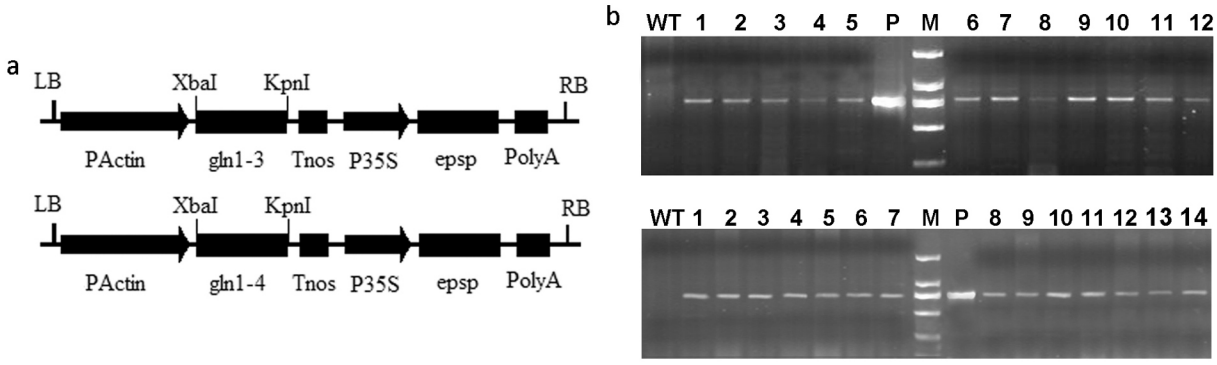

Figure 1 - The T-DNA region of the plasmid and the PCR results of transgenic lines. a) T-DNA region of the plasmid p1300-PActin::Gln1-3-p35S::epsp and p1300-PActin::Gln1-4-p35S::epsp. PActin, rice actin1 promoter; P35S, cauliflower mosaic virus (CaMV) 35S promoter; *Gln1-3* and *Gln1-4*, two members for of the cytosolic glutamine synthetase gene family; epsp, gene for the 5-enolpyruvyl-shikimate-3-phosphate synthase; TL, left T-DNA border; TR, right T-DNA border. b) Part of analysis by polymerase chain reaction (PCR) of the expression of *Gln1-3* or *Gln1-4* in transgenic lines. Specific PCR products of about 750 bp were detected in the Gln1-3 or Gln1-4 transgenic lines (lanes 1-14). P: plasmid; M, DL2000 marker.

pollination for 14 days) samples were ground with an ice-cold motor in 50 mM potassium phosphate (pH 7.8) extraction buffer. Extracts were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatants were used to assay protein content and GS activity.

Photosynthesis analysis

To determine the effects of Gln1-3 and Gln1-4 overexpression on photosynthesis, the net photosynthetic rate of maize plants before and after filling stage was measured with a portable gas-exchange system (LI-COR 6400, LI-COR, USA). As two leaves on either side of the ear provide a good indication of the source-to-sink transition during grain filling (Prioul and Schwebel-Dugue, 1992), the leaf below the ear was selected for measurement with the ambient CO_2 concentration at 400 µmol CO_2 mol^{-1} between 9:00 and 11:00 a.m. During measurements, the actual leaf temperature was 28.0 ± 1.0°C. The photosynthetic PFD (photon flux density) was maintained at 800 µmol m^{-2} s^{-1} with an internal 6400-02 BLED Source (LI-COR, USA), and the flow rate was set at 500 µmol s^{-1}.

Chlorophyll determination

Approximately 0.1 g leaves from maize below the ear before and after filling stage was homogenised with 80% acetone (v/v), and the total amount of chlorophyll was determined by spectrophotometry according to Arnon (1949).

Field trials

The field experiment was performed in the Experimental Field of Dezhou Qihe in ShanDong province (116°28'E, 36°4'N) on June 15, 2011 and JiNan in ShanDong province (117°00'E, 36°40'N) on April 12, 2012. The trial plots were arranged in a random complete block design with three replications. Forty seeds of each homozygous T3 overexpressing line and WT were sown in a double row plot in May. The plot was 1.2 × 5 m with 0.25 m intervals between plants. Plants were thinned at the five-leaf stage, resulting in 20 plants in each plot. The plant density was 67,500 ha^{-1}. When the plants reached maturity, the ears were harvested, dried and weighed to determine the hundred-grain weight. The number of ears and plants per row were recorded at harvesting. The fresh and dry weights of ears in each row were measured to determine the yield for each plot.

Statistical analysis

In measuring the physiological parameters, three or four replications were performed. All data are presented as mean ± standard deviation (SD). Comparisons between transgenic and WT were performed using the Student's t test. Values of $0.01 < p < 0.05$, $p < 0.01$ were considered statistically significant. All statistical analysis was done using Sigmaplot 11.0.

Results

Generation and PCR identification of T1 transformants

The WT plant material was an elite maize inbred line DH9632 from China. Plasmids p1300-PActin::Gln1-3-p35S::epsp and p1300-PActin::Gln1-4-p35S::epsp (Figure 1A) were transformed into maize wounded shoot tip meristems by Agrobacterium (He et al, 2013). As the T0 generations resulting from this transformation method are mostly chimeric, T0 transformants were only selected by herbicide. T1 generation plants were screened for resistance to glyphosnate herbicide and confirmed by PCR. Because there were endogenous *Gln1-3* and *Gln1-4* genes in wild type plants, PCR primers PActin-Gln1-3 and PActin-Gln1-4 (Table 1) were designed to specifically amplify

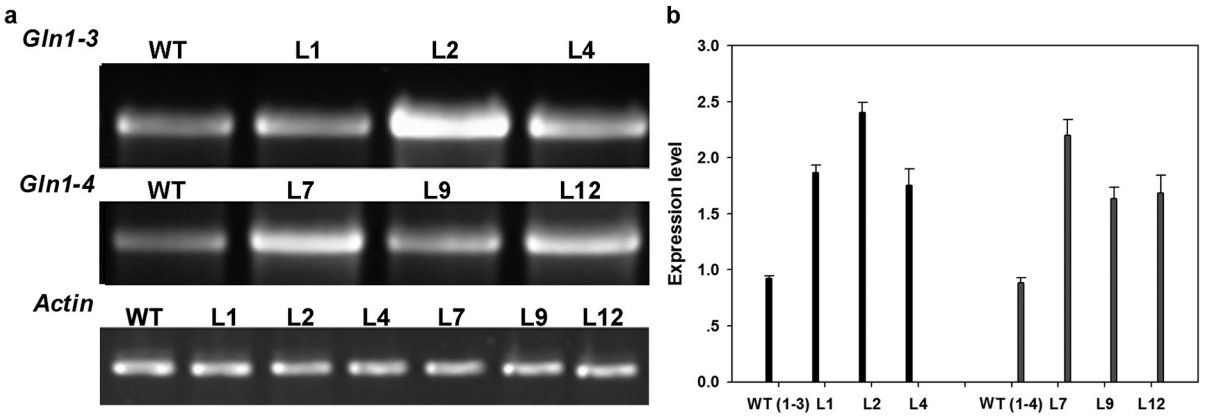

Figure 2 - Overexpression of the *Gln1-3* and *Gln1-4* genes in transgenic lines. a) Changes in expression levels of *Gln1-3* and *Gln1-4*. b) Under normal plant conditions, the expression of the *Gln1-3* and *Gln1-4* genes in transgenic lines showed significantly higher compared to WT. Values are means ± standard deviation (n = 3-5).

the Actin 1 promoter and Gln1-3 or Gln1-4 genes. As a result, 21 Gln1-3 and 25 Gln1-4 T1 transgenic lines (resulting from different transgenic events) contained the 1kb target fragments (Figure 1b) and were herbicide resistant.

Over-expression of Gln1-3 or Gln1-4 increased GS activity in T2 plants

Along with WT, six positive transgenic lines over-expressing Gln1-3 or Gln1-4 were selected to assay transcription by semi-quantitative RT-PCR and qRT-PCR. The results indicated that Gln1-3 transgenic lines (L1, L2, L4) and three Gln1-4 transgenic lines (L7, L9, L12) showed significantly higher expression of transgene compared to WT. L2 and L7 had the highest transgene expression, which was 1.5 - 2.5-fold higher than that of WT. The transgenics L1 and L9 had the lowest increases in target gene expression (Figure 2).

Three transgenic lines expressing Gln1-3 or Gln1-4 (a total of six T2 transgenic lines) and WT were used to determine GS activity in the leaves below the ear after pollination for 14 days. All of the transgenic lines showed higher GS activity, ranging from 1.5- to 1.8-fold higher than WT. The enhanced GS activity was correlated with overexpression of Gln1-3 or Gln1-4 transcripts (Figure 3). For instance, L2 had the highest Gln1-3 expression level, and its GS activity was the highest among the transformants. Similarly, the L7 transformant had the highest Gln1-4 expression and the GS activity was the highest among the transformants. L1 and L9 had the lowest up-regulation of transgene transcripts and, consequently, the lowest increase in GS activity in maize leaves.

Effect of Gln1-3/Gln1-4 overexpression on photosynthesis and chlorophyll content

T3 transgenic lines and WT were grown in normal fertilisation conditions. All six transgenic lines showed phenotypes similar to the WT plants, and there were no significant differences in plant or ear height. The photosynthesis rate and chlorophyll content were determined for the leaf below the ear between 9:00 am and 11:00 am before pollination and 14 days after pollination. There were no significant differences between the transgenic lines and WT plants of photosynthesis rate and chlorophyll contents before filling stage (Figure 4). However, after pollination for 14 days, both the photosynthesis rate and chlorophyll contents of all transformants were higher than those of WT, and L2 and L7 showed the highest levels of the Gln1-3 and Gln1-4 transformants, respectively. Gln1-3 and Gln1-4 overexpression may have up-regulated photosynthesis in leaves by promoting chlorophyll synthesis during the grain-filling stage.

Increased GS activity resulted in improved grain yield

We determined the changes in yield-related traits in field trials (Table 2). There were no obvious differences between the transgenic lines and WT in grain rows, but the former had larger grain numbers per

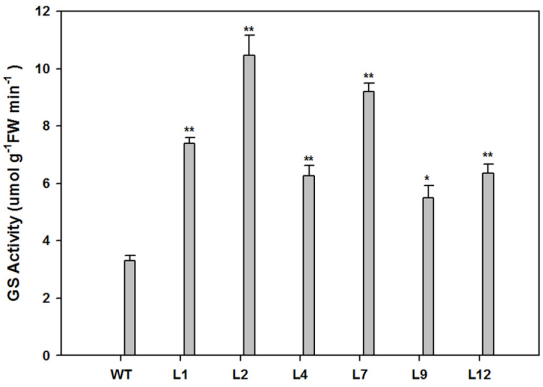

Figure 3 - Changes in the GS activities of WT and transgenic lines before and after pollination for 14 days under normal plant conditions. FW, fresh weight. All of the transgenic lines showed higher GS activity, ranging from 1.5- to 1.8-fold higher than WT. Values are means ± standard deviation (n = 3-5). An asterisk indicates significant differences from the WT at *P < 0.05 or **P < 0.01 by t test.

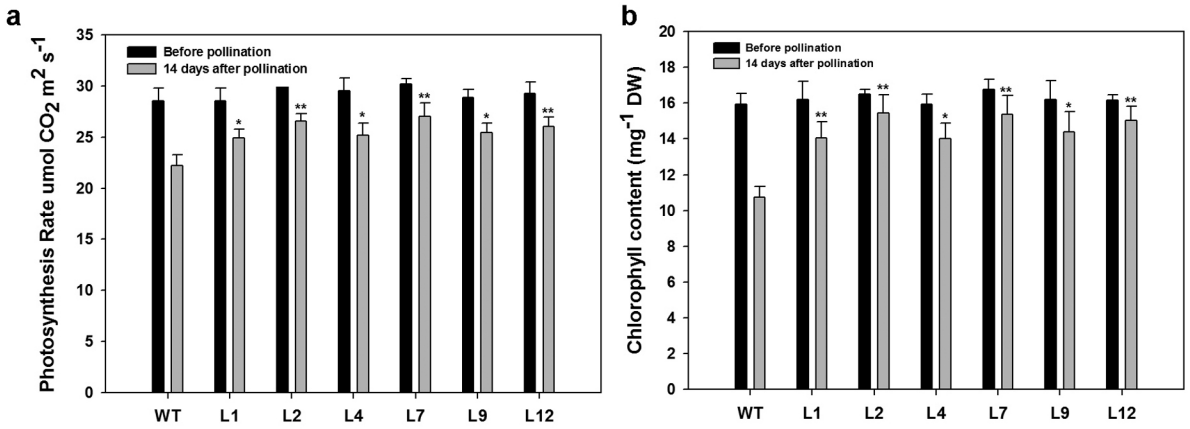

Figure 4 - Changes in the amount of chlorophyll. a) net photosynthesis, b) of WT and transgenic lines under normal conditions. Values are means ± standard deviation (n = 3-5). An asterisk indicates significant differences from the WT at *P < 0.05 or **P < 0.01 by t test.

row. Compared with WT, the grain numbers per row of Gln1-3 transformants L1, L2, and L4 were 8.9% to 12.3% higher, and the 100-grain weights were 2.2% to 3.1% heavier. The 100-grain weights of L7, L9 and L12 were 8.4% to 10.8% greater than WT (23.58 ± 1.80 g). Despite these differences, there were no visible phenotypic changes between the transgenic lines and WT. Interestingly, the transgenic dry ear weights per plot replication were significantly higher than in WT, and all transgenic lines were similar in their plot yields and dry ear weights. For instance, the highest plot yield of approximately 12.97 kg, which was 1.2-fold the yield of WT, was produced by the Gln1-3 transformant L2. The highest plot yield from a plant overexpressing Gln1-4, L7, was approximately 12.90 kg, which was also 1.2-fold the yield of WT (11.15 kg).

Discussion

High yield is the ultimate goal of crop breeding. To increase yields, various genetic or gene-engineering methods have been used to improve the kernel number and kernel weight, the two traits that significantly influence yield (Cazetta et al, 1999; Gallais and Hirel, 2004). In the single or double maize mutants, *Gln1-3* and *Gln1-4*, GS activity is significantly decreased in leaves at the vegetable stage, and the dry matter content of ears and kernel production are markedly reduced (Martin et al, 2006). In our study, transgenic plants overexpressing Gln1-3 or Gln1-4 had enhanced yields compared with WT when grown in sufficient nitrogen. We observed an increase in kernel number in *Gln1-3* transgenics that was consistent with the previous observations of Martin et al (2006). The increase in kernel size of Gln1-4 transgenics indicates that *Gln1-3* may be required for setting the kernel number, whereas *Gln1-4* is important for kernel filling.

The different degrees of yield improvement between transgenic lines might be attributed to the variable increases in Gln1-3 or Gln1-4 transcripts. This results in a 2- to 4-fold increase in leaf GS activity in the transgenic lines (Figure 3). Due to post-transcriptional modifications and position effects (Matzke et al, 1988), much selection work is required to find stable transgenic expression lines for breeding. In this study, we generated six stable lines after screen in approximately 50 transgenic plants.

In agriculture, nitrogen fertiliser is used to boost crop production (Cassman, 1999), but because excessive fertiliser use has adverse effects on the environment, improving the efficiency of nitrogen use in maize is of great significance. Thus, varieties that are highly efficient in nitrogen utilization can be developed by genetic engineering to prevent yield loss caused by nitrogen stress. As the efficiency of nitrogen use in the transgenic lines is enhanced under sufficient nitrogen conditions, we expect a similar result in nitrogen stress conditions.

Improving photosynthesis is the most plausible route for enhanced biomass production. Murata (1981) found a highly positive correlation between potential leaf photosynthesis and maximal crop growth for many crops. In this study, we observed improved photosynthesis and chlorophyll contents at 14 DAS as well as increased green leaf area (data not shown) before harvesting. This result indicated that enhanced nitrogen use increased chlorophyll content, accelerated photoreaction, and moderately increased photosynthesis efficiency. In turn, the enhanced export of photosynthate from vegetative organs promotes photosynthesis at the reproductive stage and delays leaf senescence. However, increased photosynthesis rates may not directly influence yields in transgenic lines when other genetic factors are not altered (Long et al, 2006).

In this study, two GS genes, *Gln1-3* and *Gln1-4*, were over-expressed in maize plants. Over-expression of these genes enhanced the yields of transgenic plants compared with the WT under sufficient nitrogen supply conditions over two years and in two ar-

Table 2 - The agronomic traits of transgenic lines and the WT.

Genotype	Grain rows	Number of grain per row	Dry ear weight per replication (Kg)	Hundred grain weight (g)	Plot yields (Kg)
L1	12.57±0.01	31.44±0.45*	3.07±0.16*	24.19±0.53	12.43±2.16*
L2	12.85±0.01	33.53±0.43**	3.21±0.12**	24.30±0.58*	12.97±1.42**
L4	12.59±0.02	32.43±0.36*	3.17±0.04**	24.11±0.53	12.49±2.44*
L7	12.71±0.01	29.61±0.25*	3.28±0.11**	26.13±0.59**	12.90±1.80**
L9	12.57±0.01	29.26±1.01	3.09±0.03*	25.95±1.72**	12.38±1.32*
L12	12.58±0.02	29.37±0.68	3.12±0.06*	25.56±1.51*	12.74±1.76**
WT	12.57±0.01	28.87±1.15	2.88±0.13	23.58±1.80	11.15±1.63

*P < 0.05 or **P < 0.01 by t test

eas. Increased GS activity and photosynthesis rates in the transgenic lines indicated that N uptake was enhanced. There are large areas of nitrogen-deficient soil in which normal plants cannot grow well. Therefore, cultivating transgenic maize plants that grow in nitrogen-deficient soil has important significance. In future studies, we will investigate the metabolic pathways that determine fitness in nitrogen stress conditions, which will be useful for improving the nitrogen stress tolerance of maize. This information will hopefully lead to more efficient nitrogen use and a better understanding of the relationship between N assimilation and crop productivity, which could improve yields and fertilizer utilization for this economically important crop.

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