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Identification and Expression Analysis of Glutamine Synthetase Genes in Ramie (*Boehmeria nivea* L. Gaud)

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Abstract: Glutamine synthetase (GS) plays a fundamental role in nitrogen metabolism in higher plants. Three BnGS genes have first been isolated: one gene encoding plastid GS (*BnGS2*) and two encoding cytosolic GS (*BnGS1-1* and *BnGS1-2*) in ramie. Based on a sequence analysis and phylogenetic study, three BnGS sequences were classified into three distinct sub-families. The phylogenetic analysis showed that *BnGS2* and *BnGS1-2* were closely related to those of legumes, alfalfa (*Medicago sativa*), soybean (*Glycine max*) and bean (*Phaseolus vulgaris*). The BnGS gene expression patterns revealed that each gene exhibited similar organ specificity, but distinct transcript intensity during different vegetative processes. The relatively abundant expression of *BnGS1-1* and *BnGS2* at specific organs during different vegetative processes indicates that they have critical roles in nitrogen uptake and assimilation relating to forage and growth characteristics. The *BnGS1-2* mRNA levels were remarkably upregulated in the phloem, xylem and stems during the fiber development stage, suggesting a correlation with fiber development. Therefore, the non-overlapping transcript intensity of BnGS genes in different tissues regulates ramie growth and development during different vegetative processes.

Keywords: *Boehmeria nivea* L. Gaud, Glutamine synthetase, Vegetative process, Expression patterns, Forage characteristics

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1 Introduction

The mineral nutrient nitrogen is essential for plant growth and development and is a major limiting factor in plant productivity and crop yield. Nitrogen is involved in the production of organic compounds, such as proteins, nucleic acids, chloroplasts, and hormones. All forms of nitrogen should be converted into ammonium, which is the only inorganic N-compound that can be used directly as a precursor for the biosynthesis of organic nitrogen products and is then converted into an amide residue that is necessary for primary assimilation in nitrogen utilization. Glutamine synthetase (GS) occupies the central position in nitrogen assimilation and metabolism, which are closely regulated by the development period, organ-specific cues and exogenous factors such as illumination, nitrogen resources, salinity, CO_2 concentration, water and temperature [1-6]. In addition, regulation also exists in GS isoform synthesis processes including transcription, mRNA stability, polypeptide synthesis, targeting for subcellular localization, assembly of a subunit into an active enzyme, post-translational modification of the enzyme and protein turnover [7]. Fully understanding the biochemical functions and molecular mechanism of glutamine synthetase is essential for studying the modulation of nitrogen assimilation and metabolism, for screening the germplasm and for breeding new cultivars with higher nitrogen-utilization efficiency.

The primitive functions in nitrogen assimilation and metabolism of higher plants are carried out by two distinct isoforms: a chloroplastic form (GS2) encoded by one gene for most plants, and a cytosolic form (GS1) encoded by 3 to 5 genes, depending on the species [8]. GS2 mainly exists in leaves and correlates with the re-assimilation and metabolism of ammonium from photosynthesis and photorespiration. The GS1 isoform shows spatiotemporal specificity of expression and displays different and non-redundant roles in nitrogen metabolism throughout the entire developmental period of higher plants [9]. Studies of the mRNA expression

and protein profiles have demonstrated that all of the GS sub-families in wheat are differentially expressed in the leaves, peduncle, glumes and roots [7]. Genetic studies in rice [10-12], maize [8,13] and wheat [14] have demonstrated the colocalization of QTLs for GS protein or activity with QTLs in the mapped GS genes that are related to agronomic traits. In higher plants, *GS* influences and determines the growth and developmental periods of plants, such as the period of grain development and filling in rice [15], wheat [7] and maize [16]; the senescence process in wheat [7]; and the vegetative process in rice and barley [9,17]. In addition, GS1 activity regulates the accumulation of proline to prevent stress-induced damage, which restricts plant growth and productivity [18, 19].

Ramie (*Boehmeria nivea* L. Gaud), commonly known as "China grass", is a hardy perennial herbaceous plant of the Urticaceae family. More than 90% of all ramie plants are grown in China. Ramie is world-famous for its excellent fiber properties, such as high tensile strength, excellent thermal conductivity, coolness, ventilation, moisture absorption and antibacterial properties, and the best ramie fibers are used for textiles [20]. In addition, many studies have demonstrated that ramie could be used as an ingredient in forage proteins due to its equal nutritional value with alfalfa (*Medicago sativa*), high protein and amino acid levels, reasonable nutrition composition and high biomass [21,22]. In addition, ramie exhibits special growth characteristics, including a rapid growth rate of 4-6 cm per day during the vegetative stage. However, to date, there has been no report investigating the nitrogen metabolism pathway in ramie, and none of the genes involved in the nitrogen metabolism pathway have been identified. In addition, the molecular mechanisms of ramie growth and development and of its forage characteristics remain unknown. Therefore, the focus of our work was to isolate and characterize the members of the ramie glutamine synthetase (BnGS) gene family. Based on the sequence characteristics and expression patterns of the BnGS genes, we developed an understanding of ramie forage characterization at the molecular level and a model for the roles of specific members of the BnGS gene family during ramie vegetative processes.

2 Methods

2.1 Plant materials

The elite ramie cultivar "Zhongzhu No. 1" was grown at the experimental field of the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, China. To isolate BnGS genes, samples of tissues including leaves, roots, stems, and stem shoots, were obtained from 10-day-old seedlings; 30-day-old plants, which have a vigorous vegetative growth; and 60-day-old plants, whose fiber is approximately ripe. The sampled tissues were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation. The total RNA was equally pooled from the four tissue types during three periods of cDNA synthesis. To analyze the BnGS gene expression patterns, different tissues of three replicates, including leaves, roots, stems, stem phloem and stem xylem, were individually collected during three different vegetative processes: the seedling emergence stage (10-day-old seedlings), the fiber development stage (30-day-old plants), and the mature stage (60-day-old plants). The sampled tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

2.2 RNA isolation and cDNA synthesis

The total RNA was isolated from the tested samples using a Plant RNA Kit (Omega, USA), and cDNA was then synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. Triple-diluted cDNA was used for the following analyses.

2.2.1 Screening of potential BnGS genes from the transcriptome unigenes

In our previous work, the transcriptome sequencing of ramie "Zhongzhu No. 1" was performed at the Beijing Genomics Institute (BGI)-Shenzhen, Shenzhen, China (http://www.genomics.cn/index.php), using the HiSeq™ 2000 platform based on Illumina paired-end sequencing [23]. Therefore, *Gln1;1* and *Gln2* (GenBank Accession No. AF419608 and S69727, respectively) were used to screen the identified target unigenes of BnGS genes from the transcriptome sequencing unigenes through the software blast-2.2.17-ia32-win under the Linux operating system. The target unigenes were then further identified by NCBI BLASTn analysis.

2.2.2 Molecular cloning of the BnGS genes

The three pairs of primer sets in the flanking region of the BnGS genes were designed to clone the Open Reading Frame (ORF) region of BnGS genes using Primer 5.0: GS1-1, 5'-TCTGACTCCAAATCGTCTCTC-3' and 5'-GTTCAAACCGTCAACAAACAATC-3'; GS1-2, 5'-ATGTCGCTCCTCTCAGATCTC-3' and

5'-ATCTATGGCTTCCAGAGGATG-3'; and GS2, 5'-GAGGGAAGTTAGGAGAAAATG-3', and 5'-TATGTTCGGATTGGTTCT-3'. The 30-μL PCR amplification reaction systems contained 3.0 μL of 10X Buffer, 1.5 μL of 2.5 mmol/L dNTP, 2 μL of each primer pair (10 μmol/L), 1.5 U of Pfu DNA polymerase (TIANGEN, China), 1 μg of cDNA, and ddH_2O to the final volume. The PCR amplification products were separated by agarose gel electrophoresis and extracted with a Gel Extraction Kit (Omega, USA). The extracted target DNA was ligated into the pMD18-T vector (TIANGEN, China) and transformed into *Escherichia coli* DH5α competent cells. The recombinants were identified through blue-white color selection, and five independent positive clones were sequenced by Sangon Biotech. (Shanghai, China).

2.3 Sequence analysis and phylogenetic tree construction

The deduced BnGS subunit sequences were obtained through ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the higher plant GS isoforms that were used in the phylogenetic tree construction were collected from the NCBI GenBank database. The sequences were analyzed with MEGA 5.05 software and aligned with ClustalW. The phylogenetic tree was obtained with Neighbor-Joining methods, and a bootstrap analysis with 100 replicates was performed to assess the statistical reliability of each branch. The PROSITE software (http:/prosite.expasy.org) was used to predict the protein motif features. The SMART program (http://smart.embl-heidelberg.de/) was used to predict the protein conservative domain. The ChloroP 1.1 program (http://www.cbs.dtu.dk/services/ChloroP/) was used to predict the chloroplast transit peptide.

2.4 Quantitative real-time PCR (qRT-PCR) analysis

The BnGS genes expression patterns were evaluated by quantitative real-time PCR (qRT-PCR). qRT-PCR for *18S-rRNA* was performed in parallel as the reference gene to for normalizing the *BnGS* cDNA levels in the initial non-uniform samples [24]. The qRT-PCR reaction systems and thermal protocols were performed with the 2X SsoAdvanced SYBR Green Supermix (Bio-Rad, USA) in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) following the manufacturer's recommendations. Briefly, the reaction systems contained 2 μL of diluted cDNA, 10 μL of 2X SsoAdvanced SYBR Green Supermix and 0.4 mmol/L of each qRT-PCR primer pair. Matching primer pairs were designed for each gene according to Primer 5.0 software, and they are shown in Tab. 1. The qRT-PCR thermal protocols were as follows: 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 57°C. The melting curve of the PCR products was generated from 65 to 95°C in 0.5°C increments with 8 sec/step. The relative expression levels of the BnGS genes were analyzed using Lepta-1.0 software with normalization using the reference gene *18S-rRNA*. In addition, typically, a 10-fold serial dilution (10^3-10^7) of vectors containing BnGS genes and *18S-rRNA* was performed to generate the standard curves, respectively. Each sample was tested in triplicate. The linear equations were obtained according to the serial C_t value and the parallel log value of the dilution fold. The PCR efficiency was calculated by inserting the slope (s) value into the equation "PCR efficiency = ($10^{(-1/s)}$)".

3 Results

3.1 Identification and analysis of the BnGS genes

A total of three BnGS genes were cloned from the ramie cultivar "Zhongzhu No. 1" through PCR amplification and sequencing at Sangon Biotech (Shanghai, China) and were named *BnGS1-1*, *BnGS1-2* and *BnGS2*, GenBank ID KJ940966, KJ940967 and KJ940968 respectively (Fig. 1 and Tab. 2). The isolated cytosolic BnGS genes *BnGS1-1* and *BnGS1-2* showed an ORF of 1071 bp encoding a 356-amino-acid polypeptide, and *BnGS2* encoded a 430-amino-acid

Table 1 Matching primer pairs of the BnGS genes and 18S-rRNA in the qRT-PCR analysis.

Primer name	Sequences (5'-3')	Number of bases	Tm (°C)	Amplification products (bp)
GS1-1-QF	ACAAACGACACAATGCTGC	19	57	250
GS1-1-QR	TTCCGCTGATGTTGACTCC	19	57	
GS1-2-QF	GTGGGCTGCTCGTTACATTC	20	57	80
GS1-2-QR	CCTGGATTGGCTTAGGATCA	20	57	
GS2-QF	CATCACTCCTTTCACTGACAAGA	23	57	80
GS2-QR	TCGATTTGCTTCGAACATCA	20	57	
18S-QF	TGACGGAGAATTAGGGTTCGA	21	57	100
18S-QR	CCGTGTCAGGATTGGGTAATTT	22	57	

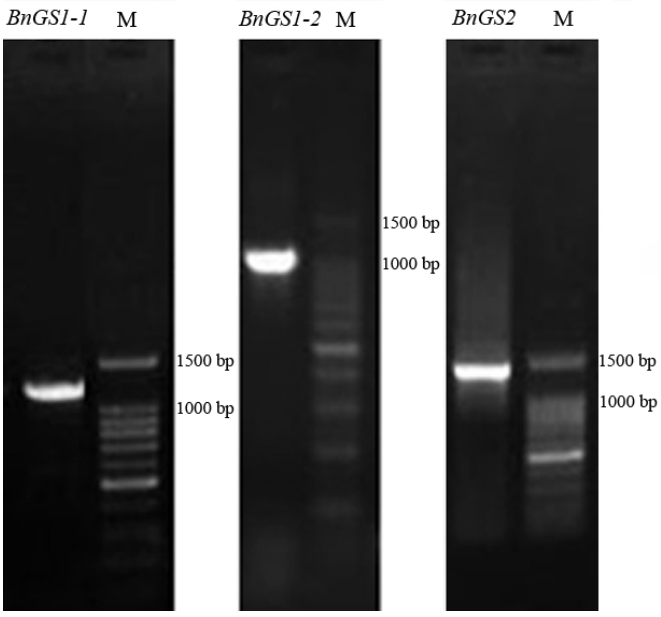

Figure 1 PCR amplification results for the BnGS genes. M: 100-bp Marker

Table 2 Details of the BnGS genes. The Mw and pI values were estimated using the web-based system at http://ca.expasy.org/tools/pi_tool.html. [a] The genes have not been released, [b] The mature peptide was estimated with a transit peptide that was 45 residues long.

Name	Accession number[a]	Length (bp)	ORF length (bp)	Number of AA	Mw (kDa)	pI
BnGS1-1	KJ940966	1205	1071	356	39.10	5.64
BnGS1-2	KJ940967	1073	1071	356	39.19	5.60
BnGS2	KJ940968	1340	1293	430	47.36	6.77
		Mature peptide[b]	1158	385	42.48	5.68

subunit transit peptide with 45 residues for transferring the subunit into the chloroplast (the nucleotide sequence of the BnGS genes were shown at supplemental data). The polypeptide Mw and pI were identical within the cytosolic and mature plastid BnGS subunits (39.10 to 42.48 KDa Mw and 5.60 to 5.68 pI) (Tab. 2).

Comparison of the nucleotide and amino acid sequences showed that the BnGS genes had identities ranging from 77.25% to 91.57% at the protein level and from 71.15% to 79.37% at the nucleotide level (Tab. 3). In addition, the sequence alignments indicate that the residues Asp-56, Cys-92, His-249, and Glu-297 are conserved in the cytosolic and plastid BnGS isoforms and that positions 303 and 368 of BnGS2 were conserved cysteine residues (Fig. 2). The sequences and structural predictions demonstrate that all of the BnGS sequence contained beta-Grasp and catalytic conservative domains that belonged to the Gln-synt domain, and that they are analogous to other plant GS isoforms. The BnGS polypeptides also contained glutamine synthetase signature 1 and an ATP-binding region (Fig. 3).

3.1.1 Phylogenetic analysis of the BnGS genes

The multi-sequence alignment analysis was carried out to examine the phylogenetic relationship between the glutamine synthetase of ramie and that of other higher plant species. A representative consequence that was produced from MEGA software analysis is shown in Fig. 4. Three ramie BnGS isoforms were grouped into three

Table 3 Comparison between the BnGS genes amino acid and nucleotide sequences using the EMBOSS Needle global alignment tool (http://www.ebi.ac.uk/emboss/align).

		Amino acid identity (%)		
		BnGS1-1	*BnGS1-2*	*BnGS2*
Nucleotide identity (%)	*BnGS1-1*		91.57	78.65
	BnGS1-2	79.37		77.25
	BnGS2	71.52	71.15	

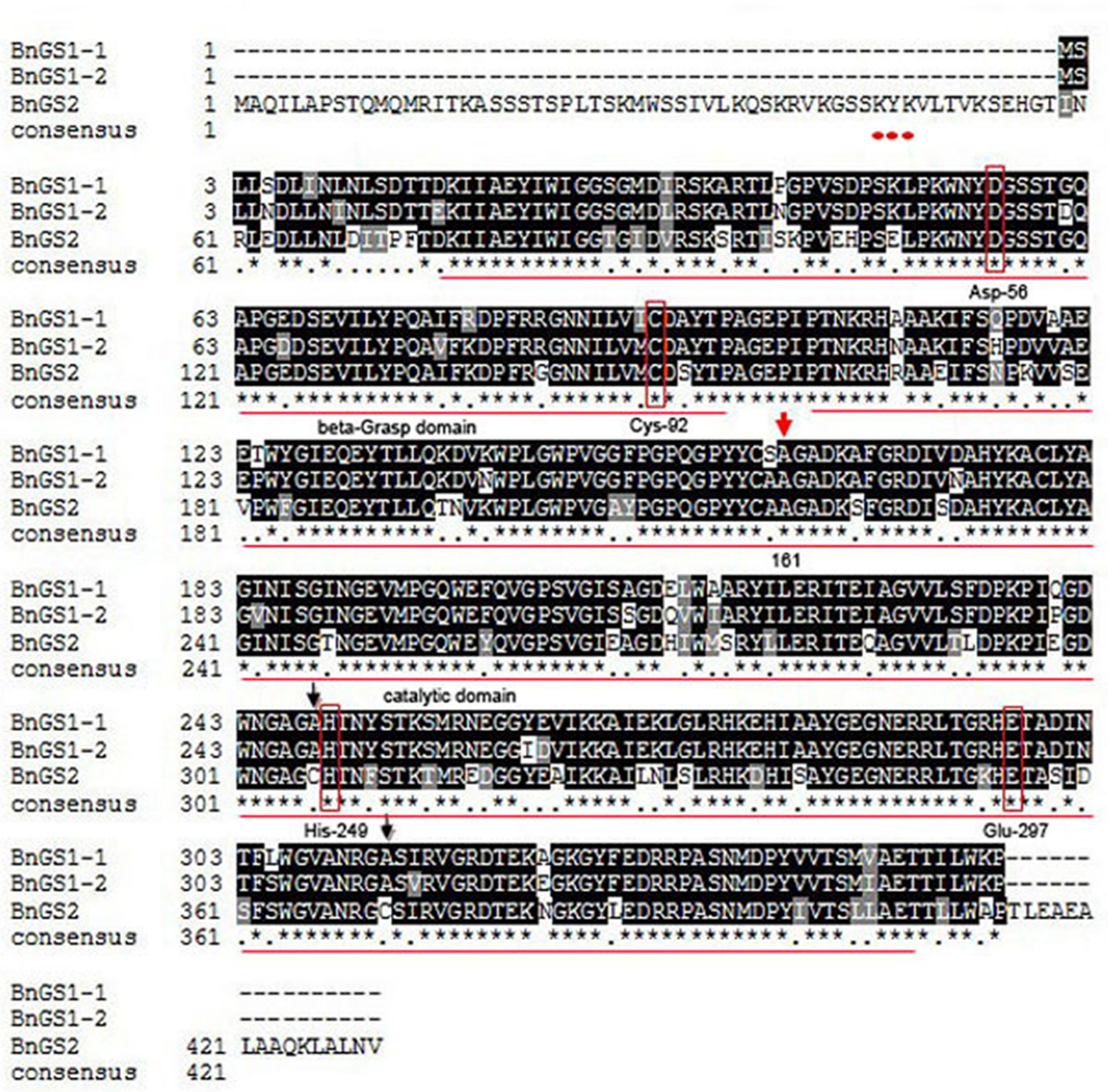

Figure 2 ClustalW multiple alignment of the BnGS amino acid sequences. The printing and shading of the multiple alignment was performed with BoxShade 3.21 at http://www.ch.embnet.org/software/BOX_form.html. The red dots indicate the start sites of mature BnGS2 sequence. The two black arrows indicate the two cysteine residues that are conserved in BnGS2. The residues that are conserved across all of the BnGS sequences and that are of interest are highlighted in boxes. The three red arrows indicate the residues that determine the substrate affinity of each isoform. The two functional domains are underlined in red.

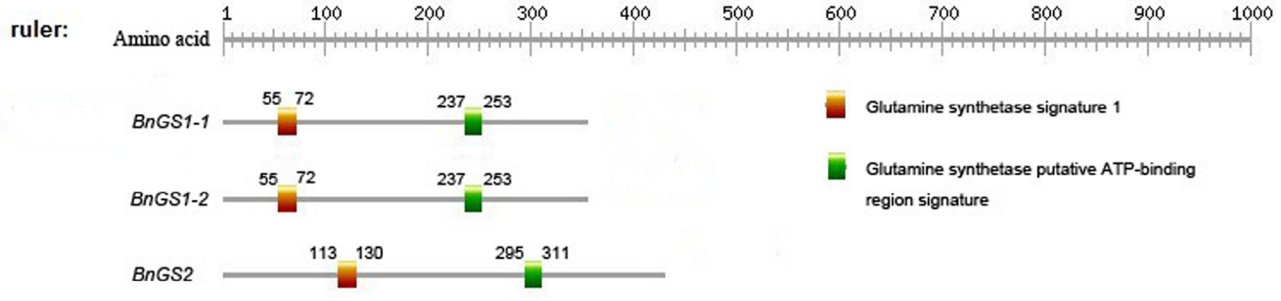

Figure 3 Identification of motifs within the BnGS isoforms using PROSITE software.

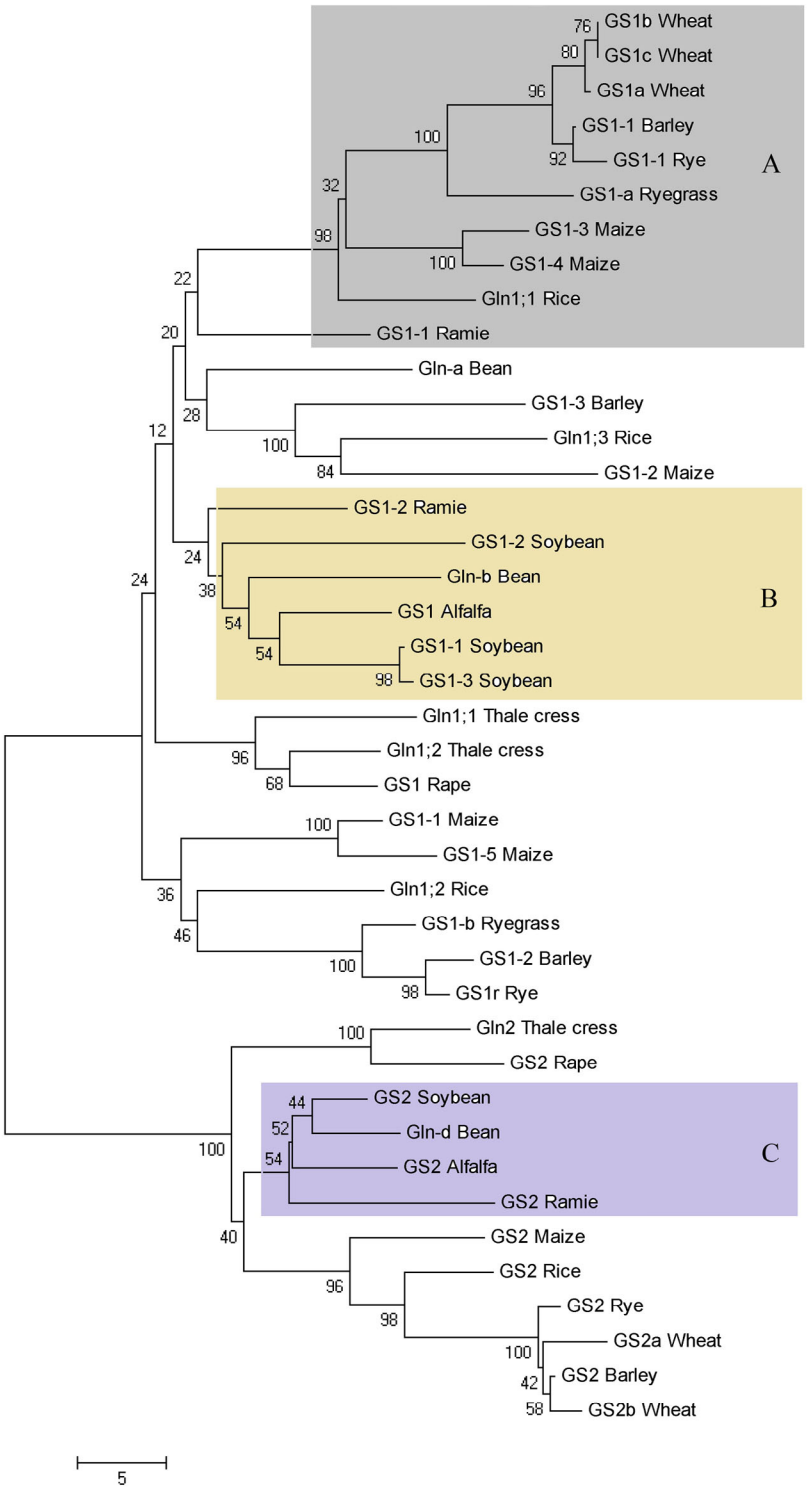

Figure 4 Phylogenetic tree of the GS protein sequences from ramie and other higher plants. The analysis was carried out using MEGA 5.05, and the tree was generated by the Neighbor-Joining method. The reliabilities of each branch point were assessed by bootstrap analysis (100 replicates). Wheat (GS2a: AAZ30060.1, GS2b: AAZ30061.1, GS1a: AAZ30057, GS1b: AAZ30058, GS1c: AAZ30059); Rice (GS2: CAA32462, Gln1;1: CAA32461, Gln1;2: CAA32460, Gln1;3: AAK18848); maize (GS2: CAA46724, GS1-1: CAA46719, GS1-2: CAA46720, GS1-3: CAA46721, GS1-4: CAA46722, GS1-5: CAA46723); Rape (GS1: CAA73063.1, GS2: CAA73062.1); Barley (GS2: CAA37643, GS1-1: JX878489, GS1-2: JX878490, GS1-3: JX878491); Thale cress (Gln1;1: NP_198576, Gln1;2: NP_176794, Gln2: AAB20558); Bean (Gln-d: CAA31234, Gln-a: CAA27632, Gln-b: CAA27631); Soybean (GS1-1: NP_001238531.1, GS1-2: XP_003544980.1, GS1-3: AAG24873.1, GS2: XP_003546121.1); Alfalfa (GS1: P04078, GS2: Q9XQ94); Ryegrass (GS1-a: ACR45959, GS1-b: ACR45960); Rye (GS1-1: AFB69879, GS1r: AFB69880, GS2: AFB69878

distinct clades, A, B, and C; one isoform represented one clade. The phylogeny of the cytosolic BnGS isoforms was very different: BnGS1-2 originated from dicots, whereas BnGS1-1 was more closely related to monocots. In dicot divergence, the ramie cytosolic BnGS1-2 was similar to those of alfalfa (*Medicago sativa*), soybean (*Glycine max*), bean (*Phaseolus vulgaris*). All of the plastid GS genes could be gathered into one clade excluding cytosolic GS. In addition, the phylogeny of GS2 isoforms rooting showed a distinct boundary between monocots and dicots. Additionally, the members of the BnGS2 sub-family included alfalfa (*Medicago sativa*), soybean (*Glycine max*) and bean (*Phaseolus vulgaris*).

3.2 Standard curves in qRT-PCR.

Standard curves for *BnGS1-1*, *BnGS1-2*, *BnGS2*, and *18S-rRNA* were obtained from analyses of the series C_t values of the 10-fold serial dilutions of vectors containing the studied genes, and these curves are shown in Tab. 4. The R^2 of the tested genes was approximately 1, at 0.9874, 0.9978, 0.9998 and 0.9899, respectively. The PCR efficiency of the tested genes ranged from 95% to 105%. In addition, the reference gene, with 101.66% PCR efficiency, behaved quite similarly to the BnGS genes, with PCR efficiencies of 97.36% for *BnGS1-1*, 102.14% for *BnGS1-2*, and 101.52% for *BnGS2*.

3.3 Spatial and temporal expression patterns of the BnGS genes

The transcript levels of the ramie BnGS genes were determined in different organs and during different vegetative processes using qRT-PCR, and the results were normalized to the reference gene *18S-rRNA*. The mRNA transcripts of cytosolic and plastid BnGS genes were detected in all of the studied tissues, as shown in Fig. 5. However, the spatial and temporal expression patterns of the BnGS genes were very distinct. *BnGS2* was primarily expressed in the leaf, and its expression patterns were regulated by the developmental stage, with the expression of *BnGS2* being more significantly activated during the seedling emergence and fiber development stages than during the mature stage. The cytosolic BnGS genes included two paralogous genes, *BnGS1-1* and *BnGS1-2*, which showed significantly different mRNA transcript levels and which displayed tissue and development diversity. During the seedling emergence and fiber development stages, *BnGS1-1* showed relatively higher levels of mRNA in the leaves, roots and phloem than in the xylem and stems. However, unlike the constitutive expression in the phloem, the *BnGS1-1* transcripts were remarkably decreased in the leaves and roots during the mature stage. *BnGS1-2* was abundantly expressed in the roots, phloem, xylem and stems, with a relatively lower expression in the leaves during the seedling emergence stage. However, during the fiber development stage, abundant *BnGS1-2* mRNA transcripts accumulated in the phloem, xylem and stems, at 30.87, 13.64 and 22.23 times the levels observed during the seedling emergence stage, respectively, and then decreased to the original levels with plant maturation. In addition, *BnGS1-1* and *BnGS1-2* displayed the same expression patterns in the roots, with high mRNA levels during the seedling emergence and fiber development stages and decreased but still relatively high expression levels during the mature stage.

4 Discussion

The details of the nucleotide and amino acid sequences of the BnGS genes in ramie allow us to identify the functions of specific BnGS isoenzymes, particularly as these details facilitate comparison with homologs from other plant species. The highlighted functional residues in the ramie BnGS polypeptide sequences are also conserved in many other plants. The highlighted functional residues of Asp-56, Cys-92, His-249 and Glu-297 have previously been reported to act as active sites and are involved in thermal stability [7, 25-27]. The conserved cysteine residues at sites 303 and 368 of BnGS2 comprise redox response sites that are important for the activity of plastid GS [7, 28]. Unno et al. indicated that the presence of Ile at position 161 was required to produce a heat-stabile GS isozyme, respectively [29]. Therefore, the ramie BnGS genes may encode isoenzymes with high activity and heat stability with specific and conserved residues in protein sequences,

Table 4 Standard curves for the 18S-rRNA and BnGS genes.

Gene name	Slope	PCR efficiency [%]	Formula	R^2
BnGS1-1	-3.3869	97.36	y = -3.3869 x + 27.542	0.9874
BnGS1-2	-3.2716	102.14	y = -3.2716 x + 31.167	0.9978
BnGS2	-3.286	101.52	y = -3.286 x + 30.76	0.9998
18S-rRNA	-3.2828	101.66	y = -3.2828 x + 24.557	0.9899

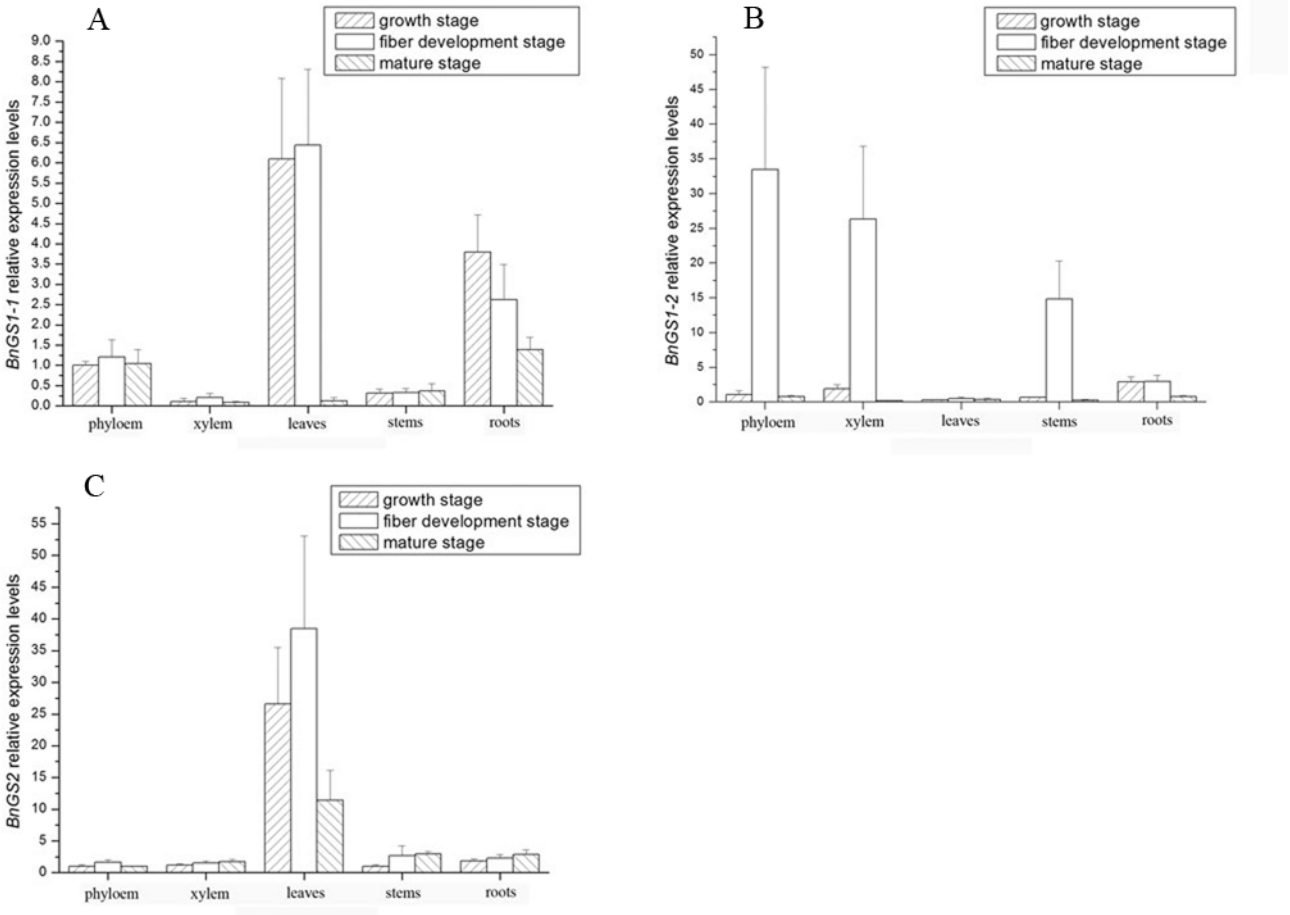

Figure 5 Spatial and temporal expression profiling of the BnGS genes through qRT-PCR analyses. Relative expression levels were analyzed using Lertpa-1.0 software normalizing with the reference gene *18S-rRNA*. Means of individual triplicate samples are shown on the graph. Error bars represent three standard errors. A: *BnGS1-1* expression profiling; B: *BnGS1-2* expression profiling; C: *BnGS2* expression profiling.

subsequently exhibiting high nitrogen-utilization efficiency and high environmental adaptability.

In previous studies, cytosolic GS from wheat [7], barley [9], soybean [30], and maize [29] was classified into three sub-families, with GS2 forming an independent clade. Similar results were obtained in this work, in which two cytosolic BnGS isoforms formed independent branches, and BnGS2 formed one clade, suggesting that BnGS genes in ramie have a different phylogenetic relationship. Similarly, in the previous report, each cytosolic GS from a monocot belonged to a specific sub-family with a monophyletic property. However, the position of cytosolic GS from dicotyledonous species was less certain [7]. In our study, two cytosolic BnGS isoforms had a fully discrepant evolution relationship: BnGS1-2 was grouped with dicots, and BnGS1-1 was closely related to homologs from monocots. Therefore, if this phylogeny of ramie cytosolic BnGS phylogeny is accurate, cytosolic BnGS from monocots and dicots may be paralogous, and this may indicate that the interspecies transfer of the gene from ancient monocots to the ancestor of ramie occurred approximately 200 million years ago [31]. This observation was also made in radish (*Raphanus sativus*) [32]. The entire plastid BnGS comprised only one clade, in which the phylogeny of plant GS2 between monocots and dicots appeared to represent two distinct subfamilies. The separation of cytosolic and plastid GS genes is due to a gene duplication 3500 Mya, pre-dating the divergence of monocots and dicots at 150 Mya [31,33], and there was no exchange of plastid GS genes during monocot and dicot evolution. In addition, BnGS1-2 and BnGS2 were closely related to the GS isoforms of *Medicago sativa*, *Glycine max* and *Phaseolus vulgaris*, suggesting that there must be certain common characteristics in nitrogen assimilation and even agronomic traits in legumes that allow ramie to possess forage characteristics and potential use as an forage grass.

Before using the reference gene to normalize the interesting genes, we validated the curves and demonstrated that the reaction efficiency was nearly identical between the reference gene and the genes of interest. In addition, the PCR efficiency of all of the genes had to be between 90% and 105% [34,35]. In this study, the correlation coefficients (R^2) were approximately 1, suggesting that the C_t value is highly correlated with the logarithm of the initial template copy number. The PCR efficiency was very similar between *18S-rRNA* and the BnGS genes, and the amplification efficiencies (97.36%-102.14%) met the qRT-PCR requirement, indicating that our qRT-PCR amplification system was optimal and that the reference gene was suitable for normalizing the BnGS genes.

The tissue- and stage-specific expression patterns and the execution of the non-redundant function of GS determine the growth and development processes of higher plants. The ramie cytosolic and plastid BnGS genes exhibiting similar organ-specific expression patterns displayed very different transcriptional concentrations, suggesting that specificity in expression intensity rather than in tissues may be a major factor controlling the extent of participation of the product of each gene during the vegetative process, which is different from thale cress [36], barley [9], and scots pine [37] but is similar to sunflower [38]. The major forms of nitrogen in the xylem sap of higher plants are glutamine and asparagine, which is synthesized from glutamine [39]. Therefore, the ammonium taken up by the roots should be efficiently assimilated within the roots by glutamine synthetase. In this study, the transcripts of cytosolic *BnGS*, *BnGS1-1* and *BnGS1-2*, were maintained at relatively high levels in the roots throughout the entire vegetative process, particularly during the seedling emergence and fiber development stages. These results strongly suggest that cytosolic BnGS genes correlate with common functions and play an essential role in the assimilation and transportation of ammonium absorbed by the roots. However, BnGS1-1 and BnGS1-2 may have non-redundant functions and localize at different positions in roots confirmed by the different expression patterns in other tissues of ramie. Goodall et al. demonstrated that both HvGS1-1 and HvGS1-2 were present within the roots tissue in barley [9]. HvGS1-1 was localized specifically to the vascular tissue of the stele, whereas HvGS1-2 was found throughout the roots and was particularly localized to the cortex and pericycle cells. In rice, OsGS1;2 is localized in the surface cells of roots and is important in the primary assimilation of ammonium ions that are taken up by the roots, with OsGS1;1 in the roots being unable to compensate for the functions of OsGS1;2 [40].

Plants primarily acquire nitrogen from the soil in the form of NO_3^-, which is sequentially reduced to NH_4^+ in the leaves by the enzymes nitrate reductase and nitrite reductase in the cytoplasm and plastid, respectively [41]. Artus et al. and Orea et al. demonstrated that the levels of NH_4^+ that were released during photorespiration were very high and that the re-assimilation of this ammonium in the plant leaves was crucial for the efficiency of nitrogen use [42, 43]. In our study, *BnGS2* was expressed abundantly in the leaves, particularly during the seedling emergence and fiber development stages. The combined results for *BnGS1-1* in the leaves and roots and *BnGS1-2* in the roots the maintenance of remarkable transcript levels of both genes during the same stages suggests that the non-overlapping transcriptional concentration of BnGS genes coordinates NO_3^- uptake and NH_4^+ assimilation to provide nitrogenous compounds for rapid ramie growth during vegetative processes. In addition, these functions are also somewhat correlated with *BnGS1-2* in the phloem and xylem, as there were relatively high levels of *BnGS1-2* mRNA in these tissues during the seedling emergence stage. In addition, *BnGS1-2* transcripts significantly increased during the fiber development stage and noticeably decreased during the mature stage in the phloem and xylem. These results indicate that *BnGS1-2* play a central role in fiber development. Rapid fiber elongation and plant growth require massive amounts of nitrogen and carbon compounds. The remarkable enhancement of BnGS activity increases the synthesis of glutamine, which is the major amino acid for further nitrogen metabolism, and, possibly in conjunction with glutamate dehydrogenase (GDH), plays a major role in controlling the translocation of organic carbon and nitrogen metabolism to maintain normal development in higher plants [44-46]. Moreover, in cereals, GS1 and GDH activities represent putative key reactions that may influence agronomic traits, such as grain yield and grain composition, from previous quantitative genetic studies [47]. Therefore, the functions of BnGS genes with non-overlapping transcriptional concentrations in different tissues regulate ramie growth and development and may display some discrepancies and complications. To date, only He et al. has reported the relative interactions between GS genes and fiber development, revealing that *GhGS* is significantly correlated with fiber strength but is not directly correlated with the accumulation of cellulose in cotton (*Gossypium hirsutum* L. *acc* 7235 and TM-1) [48]. Therefore, the finding of roles for *BnGS1-2* in ramie fiber development is a significant finding regarding the functions of *GS* in higher plants, particularly in textile crops with long fibers. In addition, considering *BnGS1-2* are

involved in fiber and xylem development, the BnGS genes in ramie that are correlated with vegetative processes and forage characteristics should be further studied.

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References

[1] Becker T.W., Carrayol E., Hirel B., Glutamine synthetase and glutamate dehydrogenase isoforms in maize leaves: localization, relative proportion and their role in ammonium assimilation or nitrogen transport, Planta, 2000, 211, 800-806

[2] Dai T., Cao W., Sun C., Jiang D., Jing Q., Effect of enhanced ammonium nutrition on photosynthesis and nitrate reductase and glutamine synthetase activity of winter wheat, J. Appl. Ecol., 2003, 14, 1529-1532, (in Chinese)

[3] Teixeira J., Fidalgo F., Salt stress affects glutamine synthetase activity and mRNA accumulation on potato plants in an organ-dependent manner, Plant Physiol. Bioch., 2009, 47, 807-813

[4] Fleury D., Jefferies S., Kuchel H., Langridge P., Genetic and genomic tools to improve drought tolerance in wheat, J. Exp. Bot., 2010, 61, 3211-3222

[5] Larios B., Aguera E., Cabello P., Maldonado J.M., De La Haba P., The rate of CO_2 assimilation controls the expression and activity of glutamine synthetase through sugar formation in sunflower (*Helianthus annuus* L.) leaves, J. Exp. Bot., 2004, 55, 69-75

[6] Nord-Larsen P.H., Kichey T., Jahn T.P., Jensen C.S., Nielsen K.K., Hegelund J.N., et al., Cloning, characterization and expression analysis of tonoplast intrinsic proteins and glutamine synthetase in ryegrass (*Lolium perenne* L.), Plant Cell Rep., 2009, 28, 1549-1562

[7] Bernard S.M., Moller A.L.B., Dionisio G., Kichey T., Jahn T.P., Dubois F., et al., Gene expression, cellular localization and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.), Plant Mol. Biol., 2008, 67, 89-105

[8] Hirel B., Gouis L.J., Ney B., Gallais A., 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., 2007, 58, 2369-2387

[9] Goodall A.J., Kumar P., Tobin A.K., Identification and expression analyses of cytosolic glutamine synthetase genes in barley (*Hordeum vulgare* L.), Plant Cell Physiol., 2013, 54, 492-505

[10] Fan C., Xing Y., Mao H., Lu T., Han B., Xu C., et al., *GS3*, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein, Theor. Appl. Genet., 2006, 112, 1164-1171

[11] Obara M., Sato T., Sasaki S., Kashiba K., Nagano A., Nakamura I., et al., Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice, Theor. Appl. Genet., 2004, 110, 1-11

[12] Yamaya T., Obara M., Nakajima H., Sasaki S., Hayakawa T., Sato T., Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice, J. Exp. Bot., 2002, 53, 917-925

[13] Gallais A., Hirel B., An approach to the genetics of nitrogen use efficiency in maize, J. Exp. Bot., 2004, 55, 295-306

[14] Gadaleta A., Nigro D., Giancaspro A., Blanco A., The glutamine synthetase (*GS2*) genes in relation to grain protein content of durum wheat, Funct. Integr. Genomics, 2011, 11, 665-670

[15] Tabuchi M., Sugiyama K., Ishiyama K., Inoue E., Sato T., Takahashi H., et al., Severe reduction in growth and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase1;1, Plant J., 2005, 42, 641-651

[16] Martin A., Lee J., Kichey T., Gerentes D., Zivy M., Tatout C., et al., Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production, Plant Cell, 2006, 18, 3252-3274

[17] Tobin A.K., Yamaya T., Cellular compartmentation of ammonium assimilation in rice and barely, J. Exp. Bot., 2001, 52, 591-604

[18] Brugiere N., Dubois F., Limami A.M., Lelandais M., Roux Y., Sangwan R.S., et al., Glutamine synthetase in the phloem plays a major role in controlling proline production, Plant Cell, 1999, 11, 1995-2011

[19] Silveira J.A., Viegas Rde A., da Rocha I.M., Moreira A.C., Moreira Rde A., Oliveira J.T., Proline accumulation and glutamine synthetase activity are increased by salt-induced proteolysis in cashew leaves, J. Plant Physiol., 2003, 160, 115-123

[20] Goda K., Sreekala M., Gomes A., Kaji T., Ohgi J., Improvement of plant based natural fibers of toughening green composites-Effect of load application during mercerization of ramie fibers, Compos. Part A-Appl. S., 2006, 37, 2213-2220

[21] Jie Y., Kang W., Xing H., She W., Screening of ramie gene resources for forage, Acta Pratacultural Science, 2009, 26, 30-33, (in Chinese)

[22] Xiong H., The potential of ramie multi-functional development and utilization, Plant Fiber Science in China, 2001, 23, 22-25, (in Chinese)

[23] Liu T., Zhu S., Tang Q., Chen p., Yu Y., Tang S., *De novo* assembly and characterization of transcriptome using Illumina paired-end sequencing and identification of CesA gene in ramie (*Boehmeria nivea* L. Gaud), BMC Genomics, 2013, 14, 125-135

[24] Ma X., Yu C., Tang S., Zhu A., Wang Y., Zhu S., et al., Cloning and tissue expression of *Actin1* gene in different fiber development phase of ramie (*Boehmeria nivea* L.), Acta Agronomica Sinica, 2010, 36, 101-108, (in Chinese)

[25] Clemente M.T., Marquez A.J., Functional importance of Asp56 from the alpha-polypeptide of *phaseolus vulgaris* glutamine synthetase - an essential residue for transferase but not for biosynthetic enzyme activity, Eur. J. Biochem., 1999a, 264, 453-460

[26] Clemente M.T., Marquez A.J., Site-directed mutagenesis of Cys-92 from the alpha-polypeptide of *phaseolus vulgaris* glutamine synthetase alters kinetic and structural properties and confers resistance to L-methionine sulfoximine, Plant Mol. Biol., 1999b, 40, 835-845

[27] Clemente M.T., Marquez A.J., Site-directed mutagenesis of Cys-92 from the alpha-polypeptide of *phaseolus vulgaris* glutamine synthetase reveals that this highly conserved

residue is not essential for enzyme activity but it is involved in thermal stability, Plant Sci., 2000, 67, 189-197
[28] Choi Y.A., Kim S.G., Kwon Y.M., The plastidic glutamine synthetase activity is directly modulated by means of redox change at two unique cysteine residues, Plant Sci., 1999, 149, 175-182
[29] Unno H., Uchida T., Sugawara H., Kurisu G., Sugiyama T., Yamaya T., et al., Atomic structure of plant glutamine synthetase: a key enzyme for plant productivity, J. Biol. Chem., 2006, 281, 29287-29296
[30] Morey K.J., Ortega J.L., Sengupta-Gopalan C., Cytosolic glutamine synthetase in soybean is encoded by a multigene family, and the members are regulated in an organ-specific and developmental manner, Plant Physiol., 2002, 128, 182-193
[31] Biesiadka J., Legocki A.B., Evolution of the glutamine synthetase gene in plants, Plant Science, 1997, 128, 51-58
[32] Watanabe A., Hamada K., Yokoi H., Watanabe A., Biphasic and differential expression of cytosolic glutamine synthetase genes of radish during seed germination and senescence of cotyledons, Plant Mol. Biol., 1994, 26, 1807-1817
[33] Kumada Y., Benson D.R., Hillemann D., Hosted T.J., Rochefort D.A., Thompson C.J., et al., Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes, Proc. Natl. Acad. Sci. USA, 90, 3009-3013
[34] Cirillo C, Accotto G.P, Vaira A.M., Vecchiati M., Acciarri N., Nervo G., Selection for resistance to tomato spotted wilt virus (TSWV) in tomato, Petria, 1999, 9, 344-345
[35] Yang L., Ding J., Zhang C., Jia J., Weng H., Liu W., et al., Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR, Plant Cell Rep., 2005, 23, 759-763
[36] Peterman T.K., Goodman H.M., Glutamine-synthetase gene family of *Arabidopsis thaliana* – light regulation and differential expression in leaves, roots and seeds, Mol G Genet, 1991, 230, 145-154
[37] Avila C., Suaarez M.F., mez-Maldonado J.O., Canovas F.M., Spatial and temporal expression of two cytosolic glutamine synthetase genes in scots pine: functional implications on nitrogen metabolism during early stages of conifer development, Plant J., 2001, 25, 93-102
[38] Montenegro M., Maldonado J.M., Perez-Vicente R., Sunflower cytosolic-glutamine synthetase genes showing similar organ-specificity patterns exhibit very different expression intensities by reverse-transcription polymerase chain reaction and Northern analysis, Protoplasma, 1999, 207, 154-157
[39] Lea P.J., Sodek L., Parry M.A.J., Shewry P.R., Halford N.G., Asparagine in plants, Ann. Appl. Biol., 2007, 150, 1-26
[40] Funayama K., Kojima S., Tabuchi-Kobayashi M., Sawa Y., Nakayama Y., Hayakawa T., Cytosolic glutamine synthetase1;2 is responsible for the primary assimilation of ammonium in rice roots, Plant Cell Physiol., 2013, 54, 934-943
[41] Lam H.M., Coschigano K., Oliveira I.C., Melo-Oliveira R., Coruzzi G.M., The molecular-genetics of nitrogen assimilation into amino acids in higher plants, Annu. Rev. Plant Physiol. Plant Mol. Biol., 1996, 47, 569-593
[42] Artus N.N, Somerville S.C., Somerville C.R., The biochemistry and cell biology of photorespiration, Crit. Rev. Plant Sci., 1986, 4, 121-147
[43] Orea A., Pajuelo P., Pajuelo E., Quidiello C., Romero J.M., Marquez A.J., Isolation of photorespiratory mutants from *Lotus japonicus* deficient in glutamine synthetase, Physiol. Plantarum, 2002, 115, 352-361
[44] Grabowska A., Kminta J., Bielawski W., Glutamine synthetase and glutamate dehydrogenase in triticale seeds: molecular cloning and genes expression, Acta Physiol. Plant., 2012, 34, 2393-2406
[45] Miflin B.J., Habash D.Z., The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops, J. Exp. Bot., 2002, 53, 979-987
[46] Paczek V., Dubois F., Sangwan R., Morot-Gaudry J.F., Roubelakis-Angelakis K.A., Hirel B., Cellular and subcellular localization of glutamine synthetase and glutamate dehydrogenase in graps gives new insights on the regulation of carbon and nitrogen metabolism, Planta, 2002, 216, 245-254
[47] Kichey T., Gouis J.L., Sangwan B., Hirel B., Dubois F., 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., 2005, 46, 964-974
[48] He Y., Guo W., Shen X., Zhang T., Molecular cloning and characterization of a cytosolic glutamine synthetase gene, a fiber strength-associated gene in cotton, Planta, 2008, 228, 473-483

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