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ARTICLE

Genome-Wide Identification and Expression Analysis of *GS* and *GOGAT* Gene Family in Pecan (*Carya illinoinensis*) under Different Nitrogen Forms

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ABSTRACT

Ammonium nitrogen (NH_4^+-N) is one of the main forms of nitrogen absorbed and utilized by plants, and mastering the regulatory mechanism of plant ammonium assimilation is a key way to improve the efficiency of plant nitrogen utilization. Glutamine synthetase (GS) and glutamate synthase (GOGAT), two key enzymes for ammonium assimilation, have rarely been studied in pecan. In this study, *GS* and *GOGAT* family members of pecan were identified and analyzed using bioinformatics methods. The results indicated that 6 *GS* and 4 *GOGAT* genes were identified. The cis-acting elements can be broadly categorized into light-responsive, hormone-responsive, and stress-responsive elements. The findings from the analysis of homologous evolution revealed that neither of the two gene families experienced tandem duplication events. Additionally, different ratios of ammonium to nitrate nitrogen were set to analyze the activities of GS and GOGAT enzymes and expression levels in pecan. The results demonstrate differences in the activities of GS and GOGAT enzymes and the gene expression levels in various tissues of pecan under different nitrogen form ratios. This study established a foundation for further mastering the molecular regulatory mechanism of nitrogen assimilation in pecan, and provided a theoretical basis for enhancing the ability of pecan to absorb and utilize nitrogen.

KEYWORDS

Pecan; NH_4^+-N; glutamine synthetase; glutamate synthase; gene family

1 Introduction

Nitrogen (N) is one of the three major elements in fertilizers, and N fertilizer plays a significant role in promoting crop yield. However, recent studies have found that the majority of N fertilizer is applied in the form of urea, which not only leads to significant nitrogen loss but also causes environmental pollution [1]. To reduce the impact of nitrogen fertilizer runoff on both plants and the environment, it is essential to adopt reasonable fertilization practices. Doing so can not only increase plant yields but also decrease environmental pollution. Plants primarily absorb and utilize nitrogen in the forms of Ammonium nitrogen (NH_4^+) and nitrate nitrogen (NO_3^-) [2], and they have varying preferences for these two forms of nitrogen. Research has shown that *Pseudotsuga menziesii* seedlings have a higher potential for absorbing NO_3^- than NH_4^+ [3], and pecans also tend to prefer NH_4^+ [2]. When inorganic N sources enter the higher

plant, they must be converted into NH_4^+ and then transformed into organic N through N assimilation [4]. In this process, glutamine synthetase (GS) and glutamate synthase (GOGAT) play important roles, forming the "GS-GOGAT cycle" [5].

Pecan (*Carya illinoensis* (Wangenh.) K. Koch), a member of the genus *Carya* in the family *Juglandaceae*, is native to Eastern North America. Pecan kernels are rich in protein and have a high oil content, with 90% of it being unsaturated fatty acids [6]. The straight trunk of the pecan tree makes its wood suitable for furniture and landscaping [7]. The pecan contains various phytochemicals [8], such as phenolic compounds. Reports indicate that pecans contain phenolic compounds and other antioxidants, which have a significant effect on degenerative diseases [9]. Due to its nutritional value, economic value, and medicinal value, pecan is widely popular both domestically and internationally [10].

GS is tasked with the conversion of NH_4^+ absorbed by plants into organic N, playing a pivotal role in the nitrogen assimilation pathway of plants [11]. Studies have also explored the relationship between GS and stresses such as drought [12]. Currently, research on members of the GS family has been conducted in a variety of plants, such as *Arabidopsis thaliana* [13], wheat (*Triticum aestivum* L.) [14], rice (*Oryza sativa* L.) [15], etc. In most plants, GS exists in two forms, including cytoplasmic GS1 and chloroplasts GS2 [16]. The cytosolic GS1 is more abundant in the companion cells of vascular tissues in plant leaves, especially in aging leaves, and it participates in the activation of N during the aging period of plant leaves, which is most significant in small grain crops [16]. GS2 is primarily involved in the assimilation of N during photorespiration and the reduction of nitrate [17]. The *GS* gene is regulated at various levels, including genetic level and transcription factors [18]. For example, the NIN-Like Protein7 (*NLP7*) transcription factor can induce the expression of the *GS2* gene [19].

GOGAT is the rate-limiting enzyme in the "GS-GOGAT cycle" [20]. GS catalyzes the conversion of NH_4^+ into glutamate, which is then further converted into glutamate again under the action of GOGAT [21]. In higher plants, GOGAT mainly includes two types: Ferredoxin-Dependent Glutamate Synthase (Fd-GOGAT) and Nicotinamide adenine dinucleotide-Dependent Glutamate Synthase (NADH-GOGAT). Different forms of the *GOGAT* gene are expressed in different plant tissues, and they also play different roles at various stages of plant growth and development [22]. Fd-GOGAT primarily functions in photosynthetic tissues, where it assimilates NH_4^+ produced by photorespiration. In contrast, NADH-GOGAT plays an important role in non-photosynthetic ammonia assimilation. There have been few studies on GOGAT in woody plants, but Cao et al. [23] for were the first to use bioinformatics methods study the GOGAT in poplar (*Populus trichocarpa* Torr. & Gray). They analyzed the response of GOGAT to carbon-nitrogen (C-N) treatment, which provides important clues for exploring the mechanism of regulating C-N balance in poplar.

During the N assimilation in process plants, GS and GOGAT play crucial roles. The gene families of *GS* and *GOGAT* have been deeply studied in many plants, but their functional research in pecan is relatively limited. This study identified 6 members of the *GS* gene family and 4 members of the *GOGAT* gene family in pecan and analyzed their physicochemical properties, gene structure, and gene duplication. This study also analyzed their expression patterns under different N forms. This research provides a solid theoretical foundation for addressing environmental pollution caused by N and improving the N utilization efficiency of pecans.

2 Methods and Materials

2.1 Plant Materials and Experimental Design

The experimental site is located at the Pecan Experimental Base of Nanjing Forestry University (30°15′50″N, 119°09′06″E). The experiment was conducted from 15 May, 2022, to 30 September, 2023. The 14-year-old 'Pawnee' pecan varieties in uniform growth conditions were selected as the experiment

material. The experiment consisted of six treatments, CK (no nitrogen application), T1 (NH_4^+:NO_3^- = 0:100), T2 (NH_4^+:NO_3^- = 25:75), T3 (NH_4^+:NO_3^- = 50:50), T4 (NH_4^+:NO_3^- = 75:25), and T5 (NH_4^+:NO_3^- = 100:0). Six trees were selected for each treatment for a total of three biological replications. The fertilizers used in the experiment were NH_4HCO_3 (17.1% N content) and $Ca(NO_3)_2$ (11% N content) at an annual rate of 700 g N per plant. Add dicyandiamide (2% DCD-N) as a nitrification inhibitor at the same time as fertilization [24].

The fertilization experiment was carried out in three stages. The first fertilization conducted in mid-May represents 50% of the total annual fertilizer application. The early June application of fertilizer constituted 30% of the yearly total fertilizer usage. The third fertilization in mid to late June accounted for 20% of the total annual fertilizer application. During the second and third fertilizations, potassium sulfate (with 50% K_2O) 150 g/plant and calcium superphosphate (with 12% P_2O_5) 625 g/plant were applied simultaneously. In the early morning of October, roots, leaves, and kernels were collected from the four cardinal directions of each tree. The collected experimental samples are placed in a laboratory refrigerator set at −80°C.

2.2 Identification of CiGS and CiGOGAT Family Members of Pecan

Firstly, all protein sequences for pecan were downloaded from the Phytozome database (Phytozome (doe.gov), accessed on 20 September 2023). Then, the hidden Markov model files for the structural domains Gln-synt_C (PF00120), Gln-synt_N (PF03951), GATase_2 (PF00310), Glu_synthase (PF01645), Glu_syn_centra (PF04898), GXGXG (PF01493), Pyr_redox_2 (PF07992), and Fer4_20 (PF14691) were downloaded from the Pfam database (Pfam is now hosted by InterPro (xfam.org), accessed on 20 September 2023) [25]. The hmmsearch program from the HMMER3.0 software suite was utilized to search through all protein sequences of pecan (E-Value < 0.001) and obtained candidate members of the gene families [26]. Next, the protein sequences of GS and GOGAT family members in *Arabidopsis* were downloaded from the TAIR database (TAIR—Home (arabidopsis.org), accessed on 20 September 2023), and then compared with the pecan sequence file (E-Value < 0.001). Afterward, the NCBI-CDD (Home—Conserved Domains—NCBI (nih.gov), accessed on 20 September 2023) was used to validate the protein sequences for their structural domains once again, ultimately determining the members of the gene family. Using the online tool ExPASy (SIB Swiss Institute of Bioinformatics | Expasy), accessed on 20 September 2023 the physicochemical properties of predicted GS and GOGAT proteins were forecast, including amino acid length (AA), molecular weights (MWs), instability index, and theoretical isoelectric points (pIs) [27]. Additionally, predictions regarding the subcellular localization of the GS and GOGAT proteins were made utilizing the Cell-PLoc 2.0 (Plant-mPLoc server (sjtu.edu.cn), accessed on 20 September 2023).

2.3 Phylogenetic Analysis of CiGS and CiGOGAT Genes

Downloaded the GS and GOGAT protein sequences from wheat, maize (*Zea mays* L.), poplar, walnut (*Juglans regia* L.), and rice in the Phytozome database (Phytozome v13 (doe.gov), accessed on 20 September 2023). Using Jones-Taylor-Thornton (JTT) model in MEGA-X software and the Neighbor-joining (NJ) method (bootstrap = 1000) to constructing phylogenetic relationships between wheat, maize, poplar, walnut, rice, *Arabidopsis* and pecan.

2.4 Conserved Domains and Conserved Motifs Analysis

Identification of conserved structural domains of GS and GOGAT in pecan was performed using Pfam. To understand the conserved motifs of GS and GOGAT protein, on online MEME (MEME—Submission form (meme-suite.org), accessed on 20 September 2023) website analysis was conducted, with the number of motifs set to 10. Then, TBtools was used to Visualize the conserved domains and motifs [28].

2.5 Analysis of Promoter Cis-Acting Elements

In order to predict cis-acting elements of pecan GS and GOGAT, the sequence is extracted from the 2.0 kb DNA upstream of the coding sequences (CDS) start site and submitted to be analyzed in the PlantCARE database (PlantCARE, a database of plant promoters and their cis-acting regulatory elements (ugent.be), accessed on 20 September 2023). Visualize the analysis results using TBtools [29].

2.6 Gene Duplication Analysis and Ka/Ks Value Calculation

Performed co-linearity analysis between pecan and walnut, and between pecan and *Arabidopsis* using the built-in plugin One Step MCScanX in TBtools [28]. To understand the evolutionary status of the *GS* and *GOGAT* genes, the ratio of non-synonymous (Ka) to synonymous substitution (Ks) rates was calculated [30].

2.7 RNA Extraction and RT-qPCR Analysis

Total RNA was extracted from pecan leaves using a Plant Total RNA Extraction Kit (Vazyme, Nanjing, China), and the cDNA was generated using the reverse transcription PCR kit (TransGen, Beijing, China). The quantitative real-time PCR (RT-qPCR) was performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The specific sequences of the primers are detailed in the Table 1. The stably expressed pecan *actin* (CiPaw.03G124400) was used as an internal reference, and the RT-qPCR parameters were as follows: 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 15 s at 60°C. The relative expression levels of the GS and GOGAT genes in pecan were determined using the $2^{-\Delta\Delta Ct}$ method [31].

Table 1: Primers for RT-qPCR

Gene name	Forward primer	Reverse primer
CiGS1.1a	AATTGACAAGCTTGGCCGGA	CGATTGGCGACACCCCATAA
CiGS1.1b	CCCAAGCCAATTCAGGGTGAT	CCTCAGCCCAAGCTTTCCAA
CiGS1.1c	TTGCCGAGGAACCCTGGTAT	AATGCCTTGTCTGCCCCTAC
CiGS1.2	CGCTAAAATCGCCTGTTGGG	ACCCGATCCACCGATCCATA
CiGS2a	CATCCGCCATTCCTGATCTGA	CCCCACATCTTTGCTGTCGT
CiGS2b	TATTGTAAGGGCTTCCCCCAC	CTGTGCCATTTTCACCTCGG
CiFd-GOGATa	GACGTGCAAGTACCGCCTT	CCAACTTTGCAACCTTCGGT
CiFd-GOGATb	GAGGAGCTTCCCGCATTTTC	CAAGTTTGCAACCCTCGGTC
CiNADH-GOGATa	TGAGCAGAAAGTTGAGGCAGA	GATTCACCCTCTTCTACCTTATTGG
CiNADH-GOGATb	GGGAATTCTAATCAGAAGGCAGA	CCTGTATTGAACACCCTCACGA
Actin	GCTGAACGGGAAATTGTC	AGAGATGGCTGGAAGAGG

2.8 Determination of CiGS and CiGOGAT Enzyme Activities

The enzyme activities of CiGS and CiGOGAT were measured using GS and GOGAT kits (Keming, Suzhou, China).

2.9 Data Analysis

Performed an analysis on the activities of GS and GOGAT enzyme, as well as the relative expression levels of genes in pecan using one-way ANOVA. Significance was analyzed using the LSD method, with

$p < 0.05$ indicating a significant difference. Performed statistical analysis the SPSS (Chicago, IL, USA, version 23.0) software. Created bar charts using GraphPad Prism 8 software.

3 Results and Analysis

3.1 Identification and Analysis of CiGS and CiGOGAT Family Members

The identification and physicochemical properties analysis of the pecan GS and GOGAT family members (Table 2) indicated that *CiGS* genes had 12 to 14 exons, while *CiGOGAT* genes had 23 to 33 exons. The CDS length of *CiGS* genes varied between 1071 and 1299 bp, while the CDS length of the *CiGOGAT* genes ranged from 4473 to 6669 bp. The sequence of CiGS1s encompasses 356 amino acids, while that of CiGS2s spans 432 amino acids. The CiNADH-GOGATs feature a range of 2222 to 2305 amino acids, and the CiFd-GOGATs contain between 1490 and 1637 amino acids The pIs of the CiGSs ranged from 5.49 to 8.07, while the pIs of the CiGOGATs ranged from 5.94 to 6.75. Most CiGSs (4/6) and all CiGOGATs (4/4) were stable proteins (instability index <40). The GRAVY value being less than 0 indicates that both GS and GOGAT proteins are hydrophilic. CiGS1.1a and CiGS1.1b are predicted by subcellular localization analysis to be positioned within the cytoplasm, CiGS1.1c and CiGS1.2 were located in chloroplast and cytoplasm, CiGS2a and CiGS2b were located in mitochondrion and the cytoplasm, and all CiGOGATs are located in chloroplast.

Table 2: Physiochemical properties of *CiGS* and *CiGOGAT*

Gene name	Exon no.	AA	CDS (bp)	MW (kDa)	pIs	Instability index	GRAVY	Subcellular localization
CiGS1.1a	12	356	1071	39.31	6.68	39.29	−0.48	Cytoplasm
CiGS1.1b	12	356	1071	39.02	5.49	36.56	−0.40	Cytoplasm
CiGS1.1c	13	356	1071	39.21	5.79	39.24	−0.42	Chloroplast Cytoplasm
CiGS1.2	13	356	1071	39.28	5.82	39.61	−0.46	Chloroplast Cytoplasm
CiGS2a	14	432	1299	47.59	8.07	45.71	−0.48	Chloroplast Mitochondrion
CiGS2b	14	432	1299	47.78	6.48	44.12	−0.50	Chloroplast Mitochondrion
CiNADH-GOGATa	23	2222	6669	244.32	6.75	36.48	−0.30	Chloroplast
CiNADH-GOGATb	23	2305	6918	254.99	6.46	36.66	−0.28	Chloroplast
CiFd-GOGATa	33	1490	4473	162.42	5.94	34.55	−0.15	Chloroplast
CiFd-GOGATb	33	1637	4914	178.25	6.23	37.12	−0.15	Chloroplast

Notes: GS: glutamine synthetase; GOGAT: glutamate synthase; Exon No.: number of exon; AA: amino acid length; CDS: coding sequence length; MW: molecular weight; pIs: isoelectric point; GRAVY: grand average of hydropathy.

3.2 Phylogenetic Analysis of CiGS and CiGOGAT

According to the phylogenetic tree (Figs. 1 and 2), the *CiGS* and *CiGOGAT* gene families were classified into three different evolutionary branches (GroupI-a ~GroupI-c). From the GS phylogenetic tree, it can be observed that the *CiGS* genes can be divided into two subfamilies: *GS1* and *GS2*. The *CiGS1* subfamily includes members *CiGS1.1a*, *CiGS1.1b*, *CiGS1.1c*, and *CiGS1.2*, whereas the *CiGS2* subfamily encompasses *CiGS2a* and *CiGS2b*. The *CiGS1* subfamily is located entirely in GroupI-a, and the *CiGS2*

subfamily is located entirely in GroupI-c. The phylogenetic tree of GOGAT shows that the *GOGAT* family can be divided into two subfamilies: *NADH-GOGAT* and the *Fd-GOGAT*, with each subfamily containing two GOGAT genes in pecan. *CiNADH-GOGAT* is located in GroupI-b, whereas *CiFd-GOGAT* is located in GroupI-c.

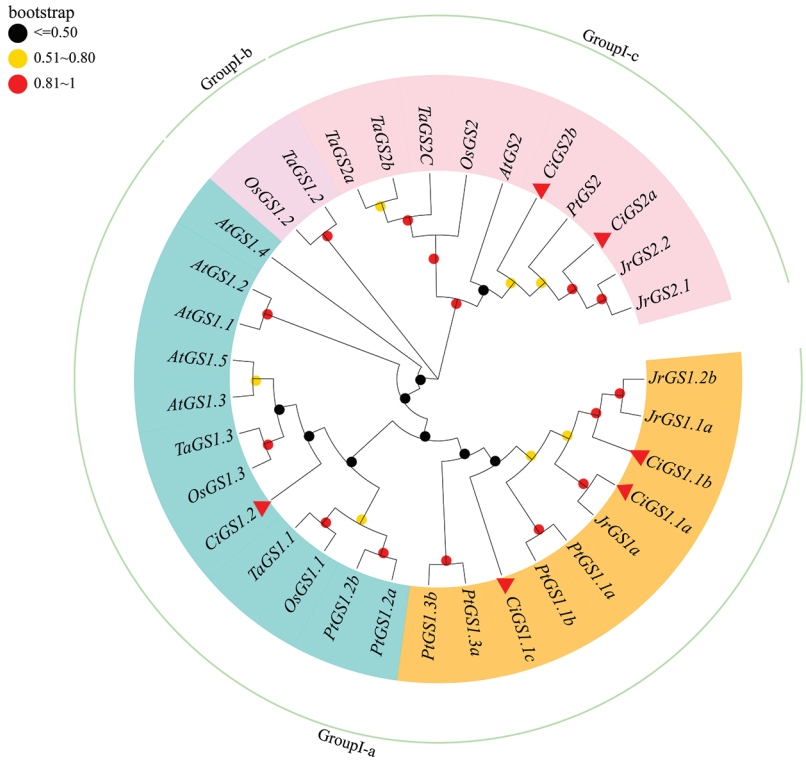

Figure 1: Phylogenetic tree of GS in *C. illinoinensis*, *A. thaliana*, *P. trichocarpa*, *T. aestivum*, *Z. mays*, *O. sativa*, and *J. regia*. Different annular sector colors represent different subfamilies. The range of Bootstrap values is displayed with circles of different colors. GS: glutamine synthetase

3.3 Conserved Motif and Conserved Domain Analysis of CiGS and CiGOGAT

The analysis of conserved motifs in GS and GOGAT in pecan (Figs. 3 and 4) has found that CiGS and CiGOGAT in pecan contain all the motifs, indicating that the protein functions of CiGS and CiGOGAT may have similarities in some aspects. The analysis of conserved structural domains revealed that the majority of CiGS1s (3/4) possessed the Gln-synt_C domain, and all CiGS2s contained the Gln-synt_N domain. The conserved structural domains of CiFd-GOGAT and CiNADH-GOGAT proteins were analysed and the results showed that CiFd-GOGAT has two less conserved structural domains than CiNADH-GOGAT, specifically Pyr_redox_2 and Fer4_20. This suggests that the protein function of CiNADH-GOGAT may be more complex than that of CiFd-GOGAT.

3.4 Analysis of Cis-Acting Elements of CiGS and CiGOGAT

Selecting the 2 kb upstream promoter regions of *CiGS* and *CiGOGAT* genes in pecan (Fig. 5) for analysis revealed that the cis-acting elements in the promoter regions of *CiGS* and *CiGOGAT* genes mainly consist of light-responsive, hormone-responsive, and stress-responsive elements. The abundance

of light-responsive elements suggests that *CiGS* and *CiGOGAT* genes are regulated by light exposure. The hormone-responsive elements in the promoter regions of *CiGS* and *CiGOGAT* genes encompass a range of triggers, including gibberellin and auxin response elements related to plant growth, as well as abscisic acid response elements related to stress response, and salicylic acid and jasmonic acid response elements related to pathogen defense. Additionally, the presence of stress-related functional elements such as low-temperature and defense stress-responsive elements indicates a significant role for *CiGS* and *CiGOGAT* genes in the plant's stress response system. Studying the cis-acting elements for *CiGS* and *CiGOGAT* genes can reveal the significant roles these proteins play in various physiological processes of plants.

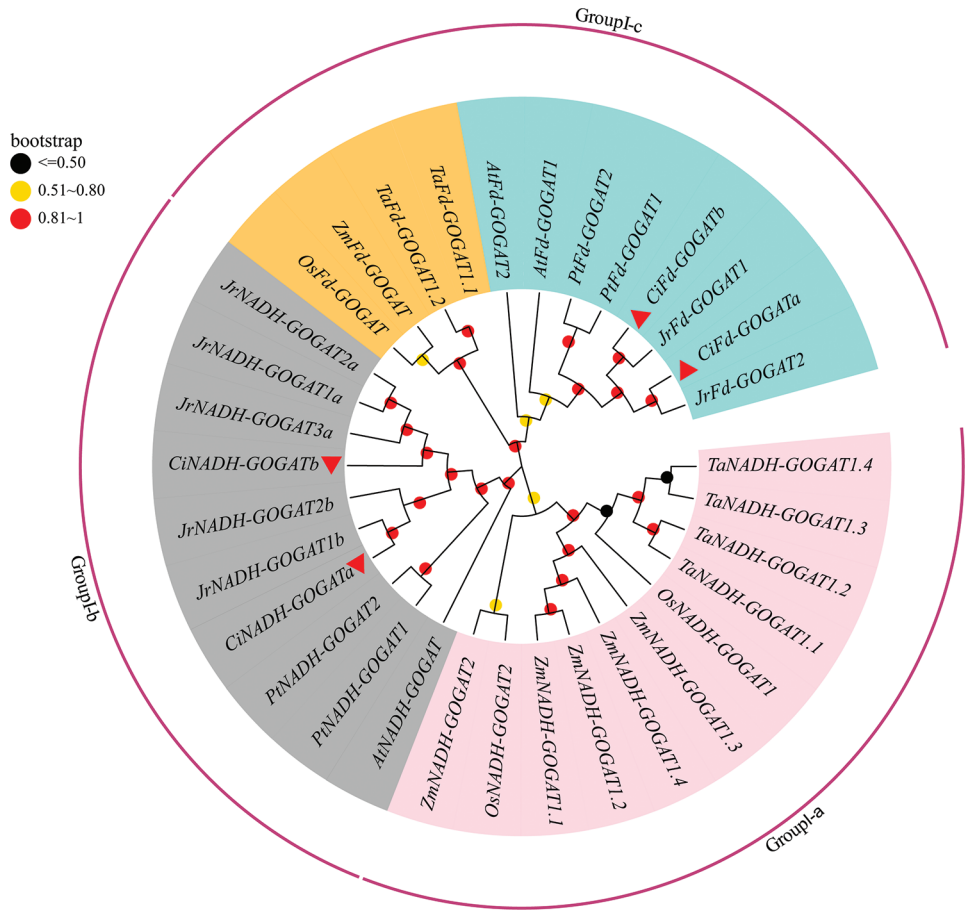

Figure 2: Phylogenetic tree of GOGAT in *C. illinoinensis*, *A. thaliana*, *P. trichocarpa*, *T. aestivum*, *Z. mays*, *O. sativa*, and *J. regia*. The range of Bootstrap values is displayed with circles of different colors. GOGAT: glutamate synthase

3.5 Duplication Events and Syntenic Analysis of CiGS and CiGOGAT

A genome-wide collinearity analysis of pecan, walnut, and *Arabidopsis* revealed that 6 *CiGS* genes from pecan are collinear with 14 *JrGS* and 10 *AtGS* genes. Additionally, 4 *CiGOGAT* genes from pecan show collinearity with 7 *JrGS* and 3 *AtGS* genes (Fig. 6). This result indicates that pecan and walnut have a relatively close phylogenetic relationship. The Ka/Ks result analysis (Table 3) indicates that the Ka/Ks ratio ranges from 0.03 to 0.13, suggesting that the members of the *GS* and *GOGAT* families have been subjected to relatively weak selective pressure, and that *GS* and *GOGAT* are under purifying selection.

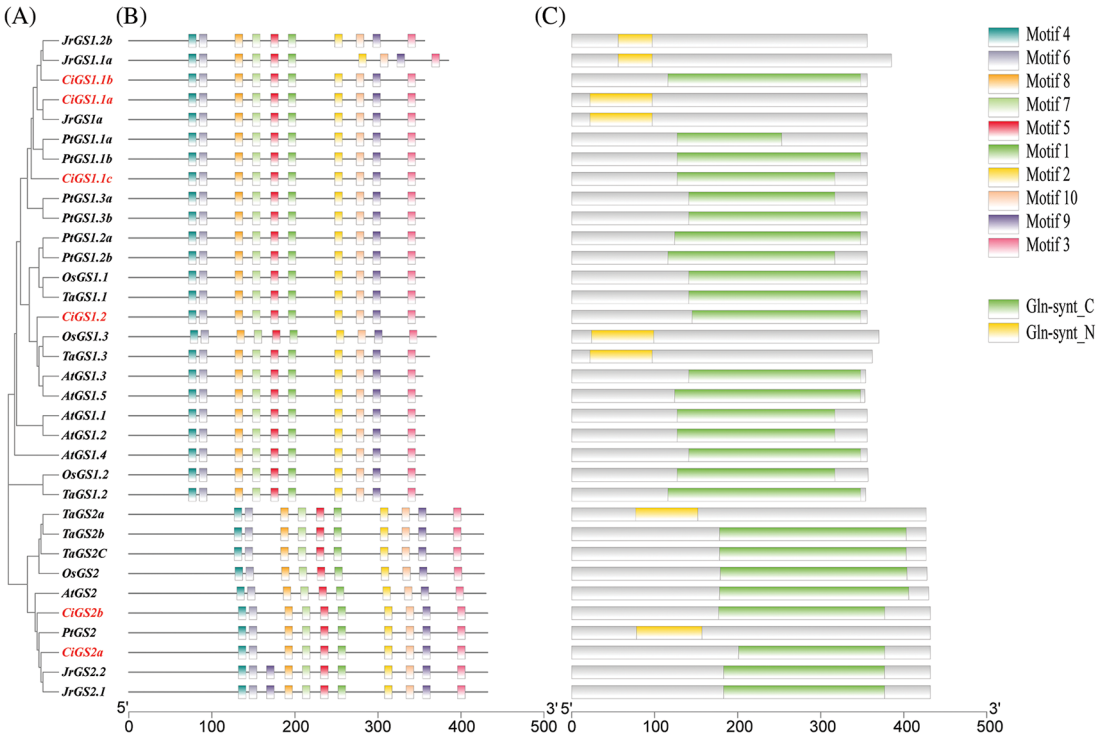

Figure 3: Conserved motif and conserved domain of GS. (A) Evolutionary tree. (B) Conserved motif. (C) Conserved domain. At: *Arabidopsis thaliana*, Jr: *Juglans regia*, Ci: *Caray illinoensis*, Pt: *Populus trichocarpa*, Os: *Oryza sativa*, Ta: *Triticum aestivum*

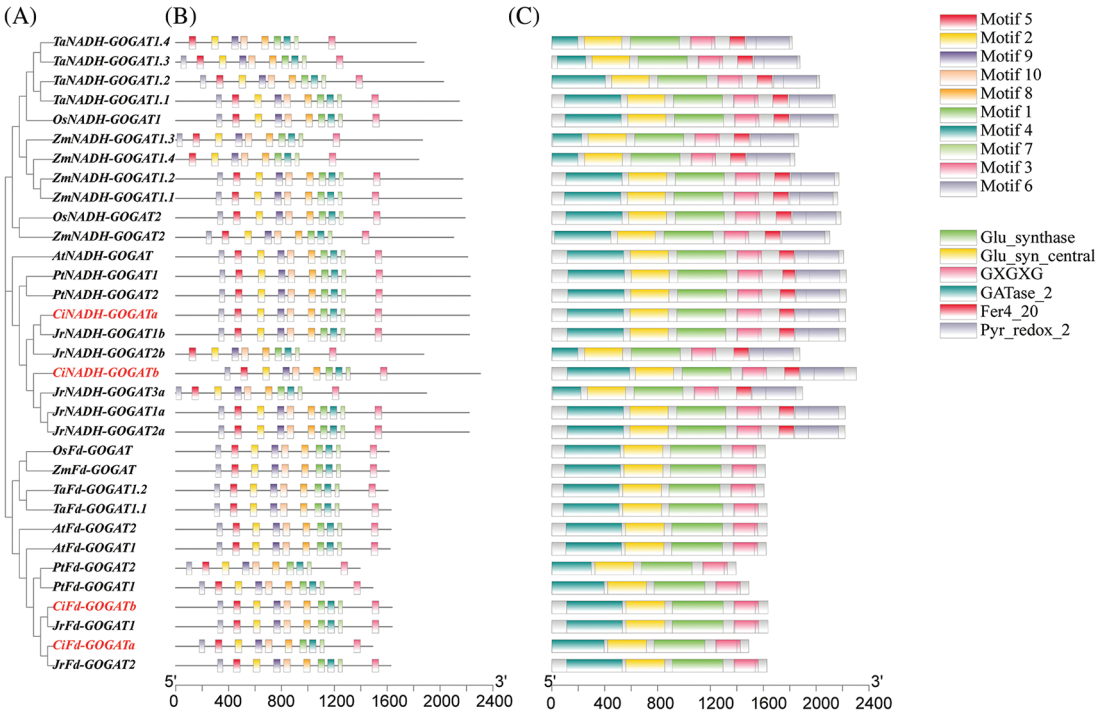

Figure 4: Conserved motif and conserved domain of GOGAT. (A) Evolutionary tree. (B) Conserved motif. (C) Conserved domain. At: *Arabidopsis thaliana*, Ta: *Triticum aestivum*, Jr: *Juglans regia*, Os: *Oryza sativa*, Ci: *Caray illinoensis*, Pt: *Populus trichocarpa*, Zm: *Zea mays*

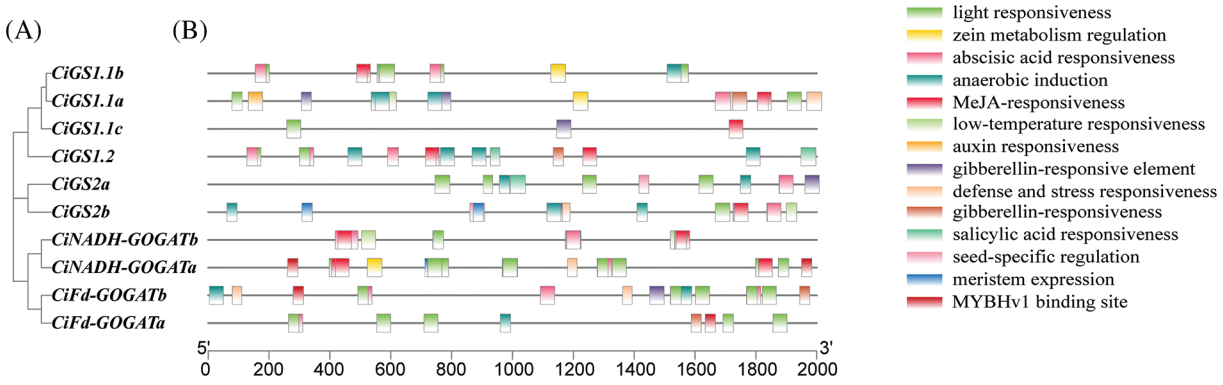

Figure 5: Distribution of cis-acting elements of *CiGS* and *CiGOGAT* genes family (A) Evolutionary tree. (B) Cis-acting elements of GS and GOGAT in pecan. Different colors indicate different Cis-regulatory element

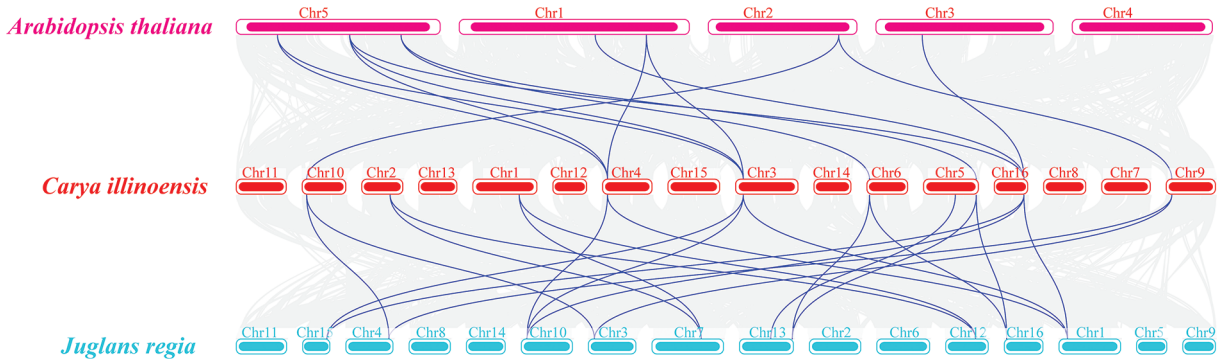

Figure 6: Pecan and walnut, as well as pecan and *Arabidopsis*, share collinear relationships among GS and GOGAT genes

Table 3: The Ka/Ks ratios for *CiGS* and *CiGOGAT*

Gene pairs	Ka	Ks	Ka/Ks
CiGS1.1a/CiGS1.1b	0.0346	0.3328	0.1039
CiGS1.1a/CiGS1.1c	0.0702	2.1672	0.0323
CiGS1.1b/CiGS1.1c	0.0628	1.6209	0.0387
CiGS1.2/CiGS1.1a	0.0678	1.0862	0.0624
CiGS1.2/CiGS1.1b	0.0689	1.0739	0.0641
CiGS1.2/CiGS1.1c	0.0905	——	——
CiGS2a/CiGS2b	0.0288	0.2568	0.1123
CiNADH-GOGATb/CiNADH-GOGATa	0.0462	0.3583	0.1291
CiFd-GOGATa/CiFd-GOGATb	0.0310	0.2917	0.1064

3.6 Effects of N Forms on CiGS and CiGOGAT Enzyme Activities of Pecan

Analysis of CiGS and CiGOGAT enzyme activities in various tissues show that were under the T4 treatment significantly increased the GS enzyme activities in roots. The NADH-GOGAT enzyme activities in roots significantly increased under T2 treatment, while it significantly decreased under T3, T4, and T5 treatments. The Fd-GOGAT enzyme activities in roots did not differ significantly among treatments. In leaves the GS enzyme activities were significantly increased under T1, T3, and T4 treatments, while T2 and T5 treatments resulted in a significant decrease in GS enzyme activities. The enzyme activities of Fd-GOGAT significantly decreased under T2, T3, and T4 treatments, while the enzyme activities of GOGAT with NADH as the electron donor significantly increased under T2 and T4 treatments. T4 and T5 treatments significantly increased the GS enzyme activities in pecan kernels, while it was significantly decreased under T2 treatment. The Fd-GOGAT enzyme activities were significantly increased under T4 and T5 treatments, and the NADH-GOGAT enzyme activities were significantly decreased under T2 and T4 treatments (Fig. 7).

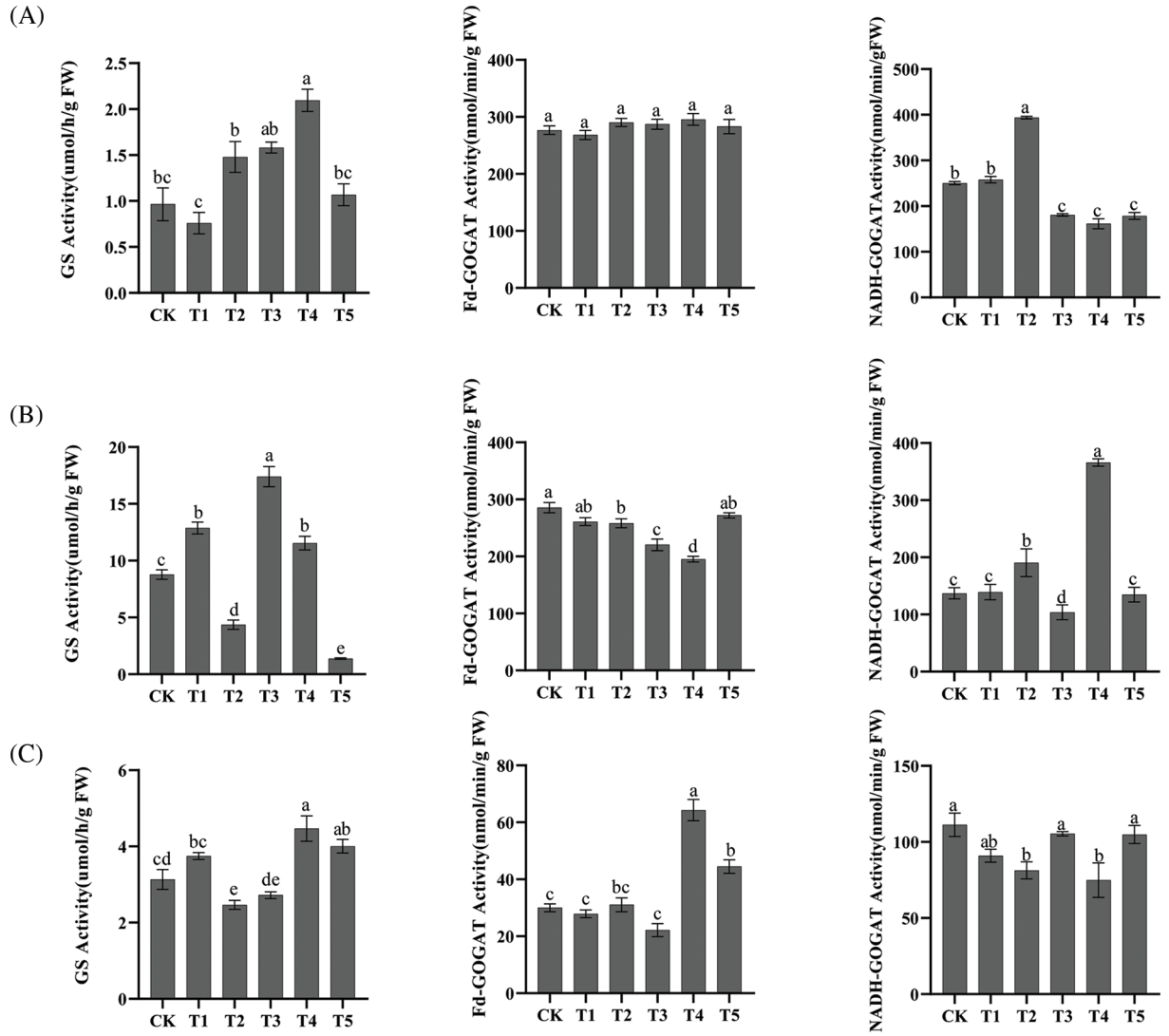

Figure 7: GS and GOGAT enzyme activities under NH_4^+:NO_3^- ratios in pecan. (A–C) Bar charts of CiGS and CiGOGAT enzyme activities in roots, leaves and kernels, respectively. Distinct lowercase letters signify statistically significant disparities among the groups ($p < 0.05$)

3.7 Effect of N Forms on CiGS and CiGOGAT Expression Levels

The expression levels of *CiGS* and *CiGOGAT* genes under N form treatment indicates that T1, T2, and T5 treatments significantly upregulated the expression levels of *CiGS1.1a* and *CiNADH-GOGATa* genes in roots. The expression levels of *CiGS1.1b* and *CiGS2b* genes were significantly upregulated under T2, T3, and T5 treatments, while the expression levels of *CiGS1.1c*, *CiGS1.2*, *CiGS2a*, and *CiFd-GOGATb* genes in roots were significantly upregulated under T2 and T5 treatments. Except for the T1 treatment, all other N form treatments significantly upregulated the expression levels of the genes *CiFd-GOGATa* and *CiNADH-GOGATb* genes in roots. In leaves, the gene *CiGS1.1a* exhibited significant increase in expression under T1, T3, and T4 treatments. A notable upregulation of *CiGS1.1b* and *CiFd-GOGATb* was observed exclusively under T4 treatment in the leaves. The *CiGS1.1c* gene showed significant upregulation across T2, T4, and T5 treatments. T2 and T4 treatments significantly upregulated the expression levels of *CiGS1.2*, *CiGS2b*, and *CiFd-GOGATa* genes, while the expression levels of *CiGS2a* gene was significantly downregulated under T3 treatment. *CiNADH-GOGATa* displayed significant upregulation under T4 and T5 treatments, and *CiNADH-GOGATb* showed a significant increase in expression under T3 treatment. In the kernels, the expression levels of the *CiGS1.1a* and *CiGS1.1b* genes were significantly downregulated in all treatments except for T2, and the expression level of the *CiGS1.1c* gene was significantly downregulated in all treatments except for T5. In the T4 treatment, the expression level of the *CiGS1.2* gene in kernels was significantly upregulated, while T2, T3, and T5 treatments significantly downregulated it. The *CiGS2a* gene showed a significant upregulated expression under T1 and T2 treatments, but a significant downregulated under T4 treatment. Expression of *CiGS2b* gene was significantly upregulated under T1 treatment. Both *CiFd-GOGATa* and *CiFd-GOGATb* genes were significantly upregulated under T3 treatment. While T1, T2, and T4 treatments all significantly downregulated the expression levels of *CiFd-GOGATb* and *CiNADH-GOGATa* genes. The expression levels of *CiNADH-GOGATb* gene were significantly downregulated under all treatments (Fig. 8).

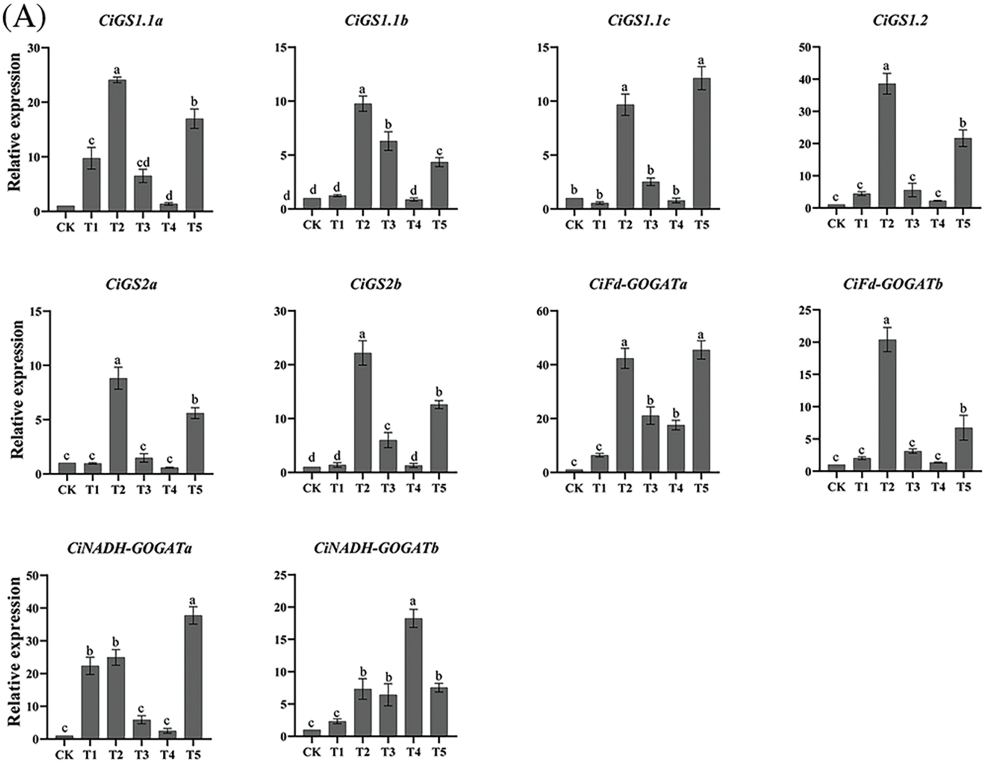

Figure 8: (Continued)

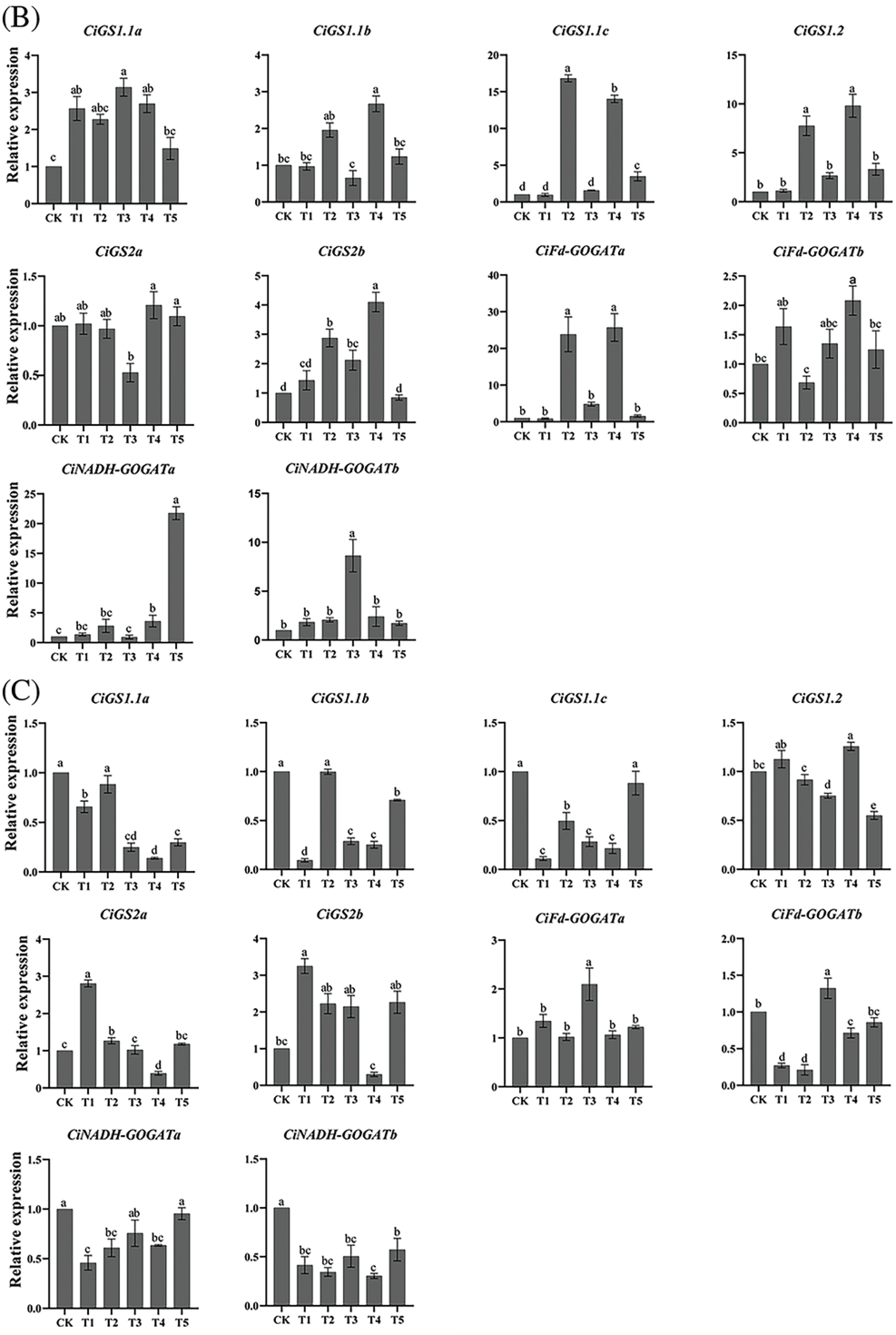

Figure 8: RT-qPCR analysis of pecan *CiGS* and *CiGOGAT* genes under varying NH_4^+:NO_3^- ratios. (A–C) Bar charts of *CiGS* and *CiGOGAT* genes expression in roots, leaves, and kernels, respectively. Distinct lowercase letters signify statistically significant disparities among the groups ($p < 0.05$)

4 Discussion

Nitrogen serves as an essential nutrient for the growth and development of plants. The supply of nitrogen has a direct impact on the plant growth rate and developmental stages. However, the metabolism of nitrogen requires the participation of multiple enzymes, among which the GS/GOGAT cycle is crucial [32]. Cytoplasmic GS1 and chloroplast GS2 have different molecular weights, *GS1* genes are 38–40 kDa and are encoded by three to five gene, *GS2* genes are 44–45 kDa and its encoding is typically the responsibility of a single gene [13]. A similar identification result was obtained for the GS family members in pecan. Motifs are conserved sequences in protein and are important parts of protein structure. Studying conserved motifs to identify the conserved domains of unknown proteins can further analyze the characteristics and functions of proteins [33]. Some studies have indicated that the function of the conserved motifs of GS have a significant role in regulating the metabolism of NH_4^+ metabolism [34,35]. CiGS2 and CiNADH-GOGAT contain more structural domains and have a higher number of introns and exons, indicating that these two genes have complex structures [36], which ultimately result in their diverse biological functions. The promoter contains specific sequences that can control gene expression, and the analysis of promoter can help to elucidate the regulatory and responsive mechanisms of gene expression [37]. The results of the cis-acting elements analysis of the GS and GOGAT family members in pecan shows that the promoter sequences of these two gene families contain multiple cis-elements related to hormone and stress responses, indicating that GS and GOGAT in pecan are involved in hormone response and stress tolerance regulation. The same situation is also found in studies on poplar [23] and wheat [38]. The number of genes for *GS* and *GOGAT* varies among different species, indicating that they may have experienced different selective pressures [39]. The collinearity results indicate that the compared with *Arabidopsis*, pecan has a higher degree of gene homology with walnut, and there are more gene duplications, suggesting that the GS and GOGAT family members of pecan and walnut are more closely related, and function similarly in the process of nitrogen assimilation.

Numerous studies on GS and GOGAT isozymes have shown that GS2 and Fd-GOGAT are predominantly present in the chloroplasts, where they primarily assimilate NH_4^+ produced by photorespiration and ammonia generated from the reduction of nitrite. NADH-GOGAT is primarily expressed in non-photosynthetic tissues, and in non-leguminous plants it may be responsible for the reassimilation of NH_4^+ from amino acid catabolism [21]. Nitrogen forms have an important influence on nitrogen metabolism in plants, especially the ammonium assimilation process [40]. In the present study, it was discovered that nitrogen form is significantly related to the GS enzyme activities in pecan. Compared to the nitrogen-deficient treatment, GS enzyme activities in roots and kernels were significantly higher under T4 treatment. In the leaves of pecan, it was observed that GS enzyme activities were significantly higher under T3 treatment, but decreased under the all-ammonium treatment, possibly due to the NH_4^+ toxicity [41]. It was also found that leaves GS enzyme activities of pecans were significantly increased under full nitrate nitrogen treatment, which may be due to the reduction of nitrate absorbed by roots and transported to the leaves to produce ammonium ions [42], which increased the concentration of the GS enzyme substrate, and consequently, the GS enzyme activities. GS enzyme activities of different species respond differently to nitrogen forms. For example, GS enzyme activities of pepper were highest under NH_4^+:NO_3^- = 25:75 and NH_4^+:NO_3^- = 37.5:62.5 [43]. In studies on the GS enzyme activity in tomato roots, it has been found that the GS enzyme activities were enhanced under the treatment of NH_4^+:NO_3^- = 50:50 [35]. It has been shown that during ammonium assimilation by most plants, NADH-GOGAT enzyme in roots and Fd-GOGAT enzyme in leaves catalyze the formation of glutamate using NADH and Fd as electron donors, respectively [44], which in turn affects plant growth and development. The GOGAT enzyme activities in leaves and kernels had opposite trends under T4 treatment, where Fd-GOGAT enzyme activities were the lowest but NADH-GOGAT enzyme activities were highest in leaves, whereas Fd-GOGAT enzyme activities were the highest but NADH-GOGAT enzyme activities were the

lowest in kernels, suggesting that the enzyme activities of the two forms of GOGAT may play complementary roles under this treatment [45]. This "GS-GOGAT cycle" is crucial in the process of ammonium assimilation in plants as GS enzyme is able to respond rapidly to nitrogen availability to provide substrate for GOGAT enzyme, and GOGAT enzyme in turn provides glutamate to GS enzyme [46].

The regulation of *GS* and *GOGAT* in plant ammonium assimilation is a complex process, and they are differentially expressed at different developmental periods and organs of the plant [47]. Isotope labeling technology can analyze the transport of a certain nutrient element within the plant body. Some studies have shown that application of ^{15}N labelled nitrogen to the roots of peanut was found to be 60-65% taken up by the roots and 30%–35% was found in the seed [48]. In poplar, *PtGS1.1* is preferentially expressed in leaves, *PtGS1.2* is more abundantly expressed in the root system of young trees, and *PtGS1.3* showed the highest expression in stems and petioles [49]. In the analysis of the expression levels of *CiGS*s and *CiGOGAT*s, it was found that the expression levels in kernels were lower than in roots and leaves, which may be due to the fact that most of the nitrogen was assimilated in roots and leaves, and only a small amount of nitrogen was transferred to the kernels for assimilation, which in turn led to organizational differences in the expression of *CiGS*s and *CiGOGAT*s. Compared to nitrogen-deficient treatments, the expression levels of *GS*s, *Fd-GOGAT*s, and *NADH-GOGATa* in pecan roots significantly increased under T2 and all-ammonium treatments. In contrast, under all-nitrate treatment, except for *CiGS1.1a* and *CiNADH-GOGATa*, the expression levels of other *GS*s and *GOGAT*s did not show significant changes, indicating that the expression of *GS*s and *GOGAT*s in pecan roots is mainly induced by NH_4^+. In addition, in pecan leaves, the expression level of *CiFd-GOGATa* significantly increased under T2 and T4 treatments, while the expression level of *CiFd-GOGATb* gene only showed a significant increase under T4 treatment. This indicates that in pecan leaves, the ammonium-dominant mixed nitrogen was more conducive to promoting the expression level of *CiFd-GOGAT* genes. However, Loulakakis et al. [50] found that nitrate had a significant induction effect on the transcription level of *Arabidopsis Fd-GOGAT*, which might be related to species-specific preferences for inorganic nitrogen. In pecan kernels, *CiGS2* genes exhibit higher expression levels compared to *CiGS1* genes, indicating that *CiGS2* genes are primarily responsible for regulating the process of ammonium assimilation within the kernels. Additionally, it was found that the *CiGS2* genes were greatly affected by the all-nitrate treatment, indicating that NO_3^-may be involved in inducing the expression of the *CiGS2* genes.

5 Conclusions

The "GS-GOGAT cycle" is the primary pathway for NH_4^+ assimilation, but its regulatory mechanism in pecan remains not fully understood. This study identified 6 *GS* and 4 *GOGAT* genes in pecan. Subsequent bioinformatics analysis along with the assessment of enzyme activities and gene expression levels under different nitrogen forms was conducted. Bioinformatics analysis can provide a better understanding of the characteristics, and functions of *GS* and *GOGAT* genes and proteins, and their roles in biological evolution. The expression of *GS* and *GOGAT* gene families in pecan is significantly affected by the ratio of nitrogen forms, and specific nitrogen source ratios can optimize the expression of these genes and the activity of related enzymes, which may enhance the plant's ability to absorb and utilize nitrogen. These results provide an important molecular basis for further study of the nitrogen assimilation mechanism in pecan and offer potential strategies for improving the nitrogen utilization efficiency of pecan and other crops by adjusting the ratio of nitrogen fertilizer.

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Availability of Data and Materials: The bioinformatics data involved in this study are provided with specific access links in the Materials and Methods section, the enzyme activity and relative expression data in this study are personal research results that cannot be publicly shared, but data will be provided according to reasonable requirements.

Ethics Approval: Not appliable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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