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Genome-Wide Analysis and Expression Profiling of *CalS* Genes in *Glycine max* Revealed their Role in Development and Salt Stress

Abstract

Abiotic stress affects plants' growth and development. Soybean is an important crop of the world, however, its production is affected by abiotic stresses. Callose Synthase is the most crucial enzyme response to environmental and developmental signals. However, in soybean, information on the callose synthase genes is limited. In this study, we analyzed the callose synthase gene family of soybean at the genome-wide scale. We also studied the genes positions, gene structure, evolutionary relations, miRNAs target sites, and expression of *CalS* genes. Resultantly 24 *CalS* genes were found in soybean, with diverse chromosomal locations, *cis*-acting elements, conserved motifs, and gene structures. Further, *GmCalS* genes were divided into four phylogenetic classes. The evolutionary classification of *CalSs* was supported by the motif and gene structure analyses. Phytohormones, abiotic stresses, and growth-responsive elements were identified in the promoter of *GmCalSs*. In addition, the *GmCalS* genes higher expression in roots, leaves, flowers, and nodules tissues provided their significance in development. Furthermore, the higher expression of *GmCalS17* and *GmCalS19* genes in response to salt stress indicated their importance against salt stress. These findings will be helpful for further investigation of the *CalS* genes in other crops.

Keywords. Callose Synthase; Expression; Growth; Regulation; qRT-PCR; Salt.

Introduction

Callose is generally found in pollen tubes, grains, cell walls, and root hairs and is essential for transporting intercellular water, cell differentiation, and development (Chen and Kim, 2009; Nedukha, 2015). However, it is present in phloem sieve plates and at cell plasmodesmata, where it can regulate the passage of molecules from one cell to another (Ellinger and Voigt, 2014). Callose Synthase is the most crucial enzyme in callose biosynthesis, with numerous transmembrane segments and a hydrophilic center loop. Furthermore, it responds to environmental and developmental signals (Granato et al., 2019). Callose is produced in many distinct places inside the plants and functions as a phloem transport regulator, significantly influencing plants' development and response to stress (Granato et al., 2019). Pollen formation, cold stimulation, mechanical injury, fungal and bacterial infection, and insect infestation alter the *CalSs* expression (Feng et al., 2021).

The *CalSs* are essential regulators in the plant vegetative growth. Barratt et al. (2011) examined in *Arabidopsis* growth retardation was due to *AtCalS9, AtCalS10,* and *AtCalS12* genes loss (Barratt et al., 2011). In addition, during the plant's vegetative growth, the *AtCalS7* mutant was responsible for the dwarf phenotype in *A. thaliana* (Barratt et al., 2011). However, callose lining loss limits the efficiency of phloem transport and stops the process of transportation assimilating, leading to the development and growth retardation (Barratt et al., 2011). Shi et al. (2015) investigated that *AtCalS5* maintained normal callose formation during development of pollen (Shi et al., 2015). Slewinski et al. (2012) reported that mutation in *Tie-dyed2 (ZmCalS)* gene was responsible for yellow leaves in maize (Slewinski et al., 2012). It was discovered that *CalS12* was responsible for synthesizing callose at pathogen attack sites (Liu et al., 2018). In addition, *Hyaloperonospora arabidopsis* and salicylic acid (SA) induce *AtCalS1, AtCalS5, AtCalS9, AtCalS10,* and *AtCalS12* expression (Dong et al., 2008). In *Citrus limon ClCalS1* gene silencing causes more susceptibility to *Xanthomonas citri* (Enrique et al., 2011). Meanwhile, *CalSs* are regulated in several signaling pathways. However, hormones and transcription factors participate in different biological regulatory mechanisms. For example, ABA treatment boosts the rice callose synthase activity, and plants resist brown planthopper (BPH) by enhancing callose deposition (Liu et al., 2017). Feng et al. (2021) found *CalSs* important role against drought, salt, heat, and, cold stress in cotton (Feng et al., 2021).

The finding of the *CalS* gene family in many plants revealed their significance in development and response to environmental stress. To date, 15 *CalS* genes have been identified in *Brassica rapa* , 7 in *Hordeum vulgare,* 12 in *Zea mays*, 8 in *Vitis vinifera,* 32 in *Brassica napus,* 12 in *Citrus sinensis*, and 12 in *Arabidopsis thaliana* (Feng et al., 2021). However, *callose synthase* genes have not been well studied in soybean. Soybeans (*Glycine max*) are important because of their economic and nutritional worth. This oil and protein-rich plant contains essential amino acids for humans and other animals. Salt and other environmental stresses pose a danger to soybean production all over the world. Thus, soybean research is crucial for enhancing food security and increasing crop yields. This work investigated chromosomal location, *cis*-acting elements, conserved motifs, gene structure, and miRNA perdition. In addition, *CalS* genes expression was observed in several tissues. Understanding how the *GmCalS* genes respond to salt stress is a foundation for investigating other *CalS* genes in salt-affected crops.

Materials and Methods

Identification of *CalS* Genes

Soybean (Glycine max Wm82.a2.v1) *CalS* genes were found using BLASTP and HMM approaches. Soybean genome sequences were obtained from the Soybean Genome Database (Schmutz et al., 2010). However, AtCalS amino acids were utilized as a query in a BLASTP search. The amino acid sequences of AtCalSs were obtained from TAIR (http://www.arabidopsis.org/) (Lamesch et al., 2012). Moreover, the HMMER 3.13 program (El-Gebali et al., 2019) was also used to search *CalS* genes. The HMM file for the CalS domain (PF02364) was obtained from the Pfam database. A total of 24 *GmCalSs* were identified in the soybean genome after screening the presence of the PF02364 domain in sequences. Data for the *M. truncatula* genome was downloaded from the Phytozome JGI 12.0 dataset (http://phytozome.jgi.doe.gov/pz/portal.html).

Physicochemical Characteristics and Subcellular Localization

We predicted the physicochemical characteristics of the GmCalS protein using the ProtParam program (https://web.expasy.org/protparam/) (Gasteiger et al., 2005). CELLO (http://cello.life.nctu.edu.tw/) version 2.5 was used to estimate the subcellular localization of GmCalS proteins. Using the TBtools program, the figure of the exons-introns configuration of *GmCalSs* was created. The MEME website was used to identify the conserved motifs of GmCalS sequences. The TBtools program was used to construct the motifs distribution.

Genes Location and Phylogenetic Analysis

The soybean genome generic feature format (GFF) file was used to determine the chromosomal position of the *GmCalSs*. The genes chromosomal locations were determined with the use of TBTools. To better understand the evolutionary relationships among *CalSs*, a phylogenetic tree was built using *AtCalSs*, *MdCalSs*, and *GmCalSs* from the three different plant species. Multiple sequence alignment was carried out using the MEGAX program (Kumar et al., 2018). In this analysis, the neighbor-joining (NJ) method was employed to build a phylogenetic tree with 1,000 bootstraps.

Synteny Analysis and Ka/Ks Ratios

Circoletto Tool (tools. bat. infspire.org/circoletto/) was used for the synteny analysis. Additionally, the ratios of Ka/Ks were calculated with the help of the KaKs 2.0 Calculator (which may be found at https://sourceforge.net/projects/kakscalculator2/). We computed the estimated divergence time for the duplicated gene pairs using the formula t = Ks/2r' and r = 6.161029 × 10^{9}) (Lynch and Conery, 2000).

Prediction of *Cis*-Regulatory Elements

The 2 kb sequences upstream of the start codons in the soybean genome were used to determine the *cis*-regulatory elements in the *GmCalS* genes promoters. PlantCARE website (Lescot, 2002) was used to analyze the promoter sequences of all *GmCalS* genes, and TBtools software was used to generate the figure.

Prediction of miRNAs

To predict miRNAs target sites, the complementary DNA sequences (CDS) of all *GmCalSs* were submitted to the psRNATarget website (https://www.zhaolab.org/psRNATarget/analysis?function=2) (Dai et al., 2018).

Expression Profiling of *GmCalS* Genes

A publicly accessible database was used for the *GmCalSs* expression analysis in different tissues. The transcriptome data of tissue expression was obtained from the NCBI SRA website with accession number SRA012188.1. The expression data in the roots, nodules, leaves, and flowers are present in Supplementary Table S6. The fragments per kilobase million (FPKM) were used to compute the transcript abundance. We used the TBtools software to generate an expression heat map.

qRT-PCR Analysis

The gene expression analysis was performed on Williams 82 variety of soybean. These seeds were germinated in a mixture of vermiculite and humus in pots for 15 days. Further, NaCl (250 mM) was applied to soybean plants for 0 hours, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours. Leaves after the treatment were put in liquid nitrogen at -80 °C for future research. Total RNA was extracted from leaves using Trizol reagent following the manufacturer's instructions (TIANGEN, Beijing, China). The PrimeScript™ RT Reagent Kit (TaKaRa, Shiga, Japan) was utilized for the cDNA synthesis using 3 μg RNA. In order to make the primers, we used Primer Premier 5. The *Actin* gene (NC_016089) was used as an internal control.

Supplementary Table S1 lists the primers used in this research, and qRT-PCR analysis was performed in three biological replicates.

Results

Identification and Characterization of *GmCalSs*

To identify genes belonging to the *CalS* family in soybean, BLASTP and HMM approaches were performed. Twelve AtCalS proteins were used as queries for the BLASTP search. Consequently, 24 *GmCalS* genes containing the *CalS* domain with Pfam ID PF02364 were found. These genes were referred to as *GmCalS1–GmCalS24*. Table 1 provides information on all 24 *GmCalS* genes. *GmCalS* genes varied in length as the number of amino acids was from 813 (*GmCalS23*) to 1965 (*GmCalS19*). The number of exons was from one (*GmCalS5* and *GmCalS17*) to fifty-one (*GmCalS12* and *GmCalS24*) (Table 1). The highest number of introns (50) was found in two genes (*GmCalS12* and *GmCalS24*), while introns were absent from two genes (*GmCalS5* and *GmCalS17*) (Table 1). The 24 GmCalS proteins were predicted with molecular weights ranging from 92.76 kDa (GmCalS23) to 227.93 kDa (GmCalS22), and their isoelectric points ranged from 7.99 (GmCalS22) to 9.6 (GmCalS13). Based on the *in silico* subcellular localization findings, nine GmCalS proteins were found on the chloroplast, and 15 GmCalS were present in the nucleus (Table 1).

CalS Genes Phylogenetic Relationships

Here, we construct a phylogenetic tree to understand the evolutionary links between the *AtCalS*, *MtCalS*, and *GmCalS* genes. *A. thaliana* contains 12 *CalS* genes and *M. truncatula* has 15 *CalS* genes (Supplementary Table S2). The *M. truncatula*, *A. thaliana*, and *G. max* CalS protein sequences were aligned to generate an unrooted phylogenetic tree. The 51 *CalSs* genes from the three plant species were divided into four groups (Figure 1). The 19 *CalSs* in Group A were as follows: 7 from *A. thaliana*, 5 from *M. truncatula*, 7 from *G. max*. Group B included 7 *G. max CalSs*, 4 *M. truncatula CalSs*, 1 *A. thaliana CalS*. However, 1 *CalSs* was found in *A. thaliana*, 2 in *M. truncatula*, and 5 in *G. max* in Group C. Furthermore, 3 *CalSs* were found in *A. thaliana*, 3 in *M. truncatula*, and 5 in *G. max* in Group D. *CalSs* in the same group may perform similar functions. Significantly, *GmCalS* genes showed consistent distribution across all groups. Group A and B had the highest number of *GmCalSs* (7), followed by C and D (5). It was also discovered that the *GmCalSs* have the strongest evolutionary ties to the *M. truncatula* species.

Gene Structures and Conserved Motifs Analysis

To understand the evolution of the soybean *CalSs*, we examined the *GmCalSs* exon-intron structures. According to the findings, introns were between 0 and 50 and exons from 1 and 51. Overall, there are two genes with a single exon and no intron; three genes with two exons and a single intron; one gene with thirteen fourteen exons; one gene with seventeen introns and eighteen exons; two genes with forty-one exons and forty introns; eleven genes with forty-two exons and forty-one introns; and one gene with forty-five exons and forty-four introns; one gene with fifty exons and forty-nine introns; one gene with fifty-one exons and fifty introns (Figure 2B). In addition, *GmCalS* gene members of the same Class had remarkably similar gene structures, consistent with the phylogenetic groups to which they belonged. In addition, the protein sequences were also analyzed to determine the motifs. The *CalS* genes have a conserved motif ranging from 4 to 10. Ten conserved motifs were found in this research, and information on these motifs can be found in Supplementary Table S3. Similar patterns of motif distribution were also seen within the group. Motifs 1, 2, 3, 4, 5, 8, and 9 were found in the *GmCalS6* gene, whereas motifs 4, 8, 6, and 9 were found in the *GmCalS23* gene. Furthermore, motifs 4, 5, 6, 8, 9, and 10 were observed in gene *GmCalS13* while gene *GmCalS5* had 1, 2, 3, 4, 5, 8, and 9 (Figure 2A). However, the *GmCalS14* gene contains 1, 2, 3, 4, 5, 6, 8, 9, and 10 motifs. It was also shown that all ten motifs were found in *GmCalS1, GmCalS2, GmCalS3, GmCalS4, GmCalS5, GmCalS7, GmCalS8, GmCalS9, GmCalS10, GmCalS11, GmCalS12, GmCalS15, GmCalS16 GmCalS17, GmCalS18, GmCalS19, GmCalS20, GmCalS21, GmCalS22, GmCalS24* genes.

Chromosomal Locations and Synteny Analysis

We determined the chromosomal position of 24 *GmCalSs* genes and found that only ten chromosomes contained *GmCalSs* genes. Most chromosomes (Chr05, Chr10, and Chr12) contained just a single gene, but Chr04 had two genes. Further, Chr06, Chr13, Chr15, and Chr18 each had three, and Chr08 had five genes (**Figure 3**). In our results, chromosomes Chr01, Chr02, Chr03, Chr07, Chr09, Chr11, Chr14, Chr16, Chr17, and Chr19 were found without any *GmCalS* gene. A study of the synteny among *G. max*, *A. thaliana*, and *M. truncatula* revealed a connection with the expression of genes and their evolution, functions, duplications, and triplications. It was discovered that the sequences of numerous *Cals* genes found in *M. truncatula* showed synteny with the *CalS* genes found in soybean. In addition, there were synteny links between the *CalS* genes of soybean and *A. thaliana* (Figure 4).

Ka/Ks Calculation

In order to calculate the molecular evolution rate, Ka/Ks for each duplicated gene pair estimated. When the Ka/Ks ratio was more than 1, it was considered that purifying selection was occurring among the duplicated genes; when it was less than 1, it was supposed that neutral selection was occurring; and when it was equal to 1, it was assumed that positive selection was occurring (Zaynab et al., 2021). Our results show that purifying selection was applied to most *GmCalS* duplicated genes during duplication. If the Ks values of *GmCalS* genes are higher than 0.52, the deviation time may be more than 100 million years ago (MYA). More intriguingly, the Ks value for the duplicated gene pair (*GmCalS5*/*GmCalS13*) was 0.632, indicating that the duplication event happened at about 51.31 MYA (Table 2).

Prediction of miRNAs

We discovered miRNAs targeting *GmCalS* genes to understand the miRNA-arbitrated post-transcriptional modification of *GmCalSs*. These miRNAs are part of different families. Supplementary Table S4 has the data of all miRNA-targeted sites/genes. According to the findings, gma-miR159 targeted a total of seven genes (*GmCalS1*, *GmCalS4*, *GmCalS12*, *GmCalS14*, *GmCalS17*, *GmCalS20*, and *GmCalS24*). The microRNAs, gma-miR172 targeted five genes *GmCalS12*, *GmCalS16*, *GmCalS19*, *GmCalS20* and *GmCalS21* genes; gma-miR171 targeted *GmCalS1*, *GmCalS3*, *GmCalS4*, *GmCalS5*, *GmCalS7*, *GmCalS9*, *GmCalS10*, *GmCalS11*,*GmCalS14*, *GmCalS16*, *GmCalS17*, *GmCalS19*, *GmCalS20*, and *GmCalS21* genes; gma-miR395 targeted *GmCalS11*, and *GmCalS22* genes; gma-miR394 targeted *GmCalS7*, *GmCalS11*, and *GmCalS18* genes; gma-miR167 targeted *GmCalS3*, and *GmCalS8* genes; gma-miR169 targeted *GmCalS2*, *GmCalS3* *GmCalS4*, *GmCalS7*, *GmCalS8*, *GmCalS11*, *GmCalS12*, *GmCalS14*, *GmCalS16*, *GmCalS17*, *GmCalS19*, *GmCalS21*, *GmCalS22*,and *GmCalS24* genes. It has been discovered that several common genes, such as *GmCalS1*, *GmCalS10*, *GmCalS12*, *GmCalS14*, and *GmCalS20*, are targeted by mostly different miRNAs.

Promoter Analysis of *GmCalS* Genes

The *cis*-regulatory elements in the promoters of *GmCalS* genes were studied to understand their regulatory functions in response to abiotic stress and during soybean growth. Supplementary Table S5 shows the *GmCalS* genes *cis*-elements details. Overall, we observed abiotic stress, phytohormones, and growth-responsive elements (Figure 5). Several abiotic stress-responsive components including anaerobic, temperatures, light, and drought were found in *GmCalS* promoters. These components include the Box 4 motif, GT1 motif, GA motif, ARE motif, MBS motif, TC-rich repeats, and LTR motif. Similarly, the TCA-element, P-box/GARE-motif, ABRE, and TGACG-motif are responsible for the responses to five different phytohormones (salicylic acid, auxin, gibberellin, methyl jasmonate, and abscisic acid). It was found that some of the elements are unique to certain genes and are distributed inconsistently. In addition, we identified four elements associated with development, including meristem expression, endosperm expression, circadian regulation and zein metabolism. These elements include the O2-site, circadian, CAT-box, and GCN4 motif, which perform a dynamic role in the various phases of growth and development of soybean. It is possible to conclude that differential gene expression for *GmCalSs* may occur during different phases of development, and abiotic stress.

***GmCalS* Genes Expression in Various Tissues**

This study utilized RNA-seq data to explore the *GmCalS* genes expression in flowers, leaves,nodules and roots. The findings demonstrated that several genes had higher expression in several tissues (Figure 6; Supplementary Table S6). The results revealed that in leaves some genes including *GmCalS5, GmCalS7, GmCalS9, GmCalS10, GmCalS11, GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS17, GmCalS18, GmCalS19, GmCalS21,* and *GmCalS24* display higher expression. However, *GmCalS3, GmCalS5, GmCalS7, GmCalS8, GmCalS9, GmCalS10, GmCalS11, GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS16, GmCalS17, GmCalS18, GmCalS19, GmCalS21,* and *GmCalS24* genes display higher expression in roots. In nodule, *GmCalS5, GmCalS7, GmCalS8, GmCalS9, GmCalS10, GmCalS11, GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS16, GmCalS17, GmCalS18, GmCalS19, GmCalS21* and *GmCalS24* genes display higher expression. Several genes showed higher expression in flower, such as *GmCalS2, GmCalS5, GmCalS7,* *GmCalS8, GmCalS9, GmCalS10, GmCalS11, GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS16, GmCalS17, GmCalS18, GmCalS19, GmCalS21* and *GmCalS24* (Figure 6; Supplementary Table S6). It was noted that certain genes demonstrated modest expression levels in various tissues (Figure 6; Supplementary Table S6). Most genes seem to have a potential role in the growth of soybean.

***GmCalS* Genes Expression Under Salt Stress Using RT-qPCR**

In this study, *GmCalS* genes expression against salt in was observed. The qRT-PCR study was conducted to analyze *GmCalS* genes expression against salt stress at various time intervals (Figure 7). According to the findings of the expression study, *GmCalS-17,* and *GmGmCalS-19* exhibited higher expression at the 12h. Further, stress-induced expression patterns give essential information on the significance of *GmCalS* genes in dealing with abiotic stress challenges.

Discussion

Plants have contact with their surroundings therefore subjected to abiotic and biotic stresses. Abiotic stress factors affect plants' anatomy, physiology, biochemistry, and morphology, significantly reducing their growth and development (Nadarajah, 2020). Several studies have reported the function of callose in development of plant and against stresses (Piršelová and Matušíková, 2013; Verma and Hong, 2001). Due to callose significance, *callose synthase* has been studied in several plants. Eight *CalSs* were identified from *Vitis vinifera*, 15 *CalSs* from *Chinese cabbage*, 12 *CalSs* from *Arabidopsis thaliana*, 7 *CalSs* from *Hordeum vulgare*, 32 *CalSs* from *Brassica napus* and 12 *CalSs* in *Citrus sinensis*. The *CalS* genes in soybean have not been described. The sequences availability of the soybean genome provides resources for identifying *CalS* genes in the soybean genome (Schmutz et al., 2010). We found 24 *GmCalS* genes, which is higher than the number of *CalS* genes in *Arabidopsis*. This is evidence of a genome duplication event in the evolutionary process of *G. max*. Gene structure analysis demonstrated that genes from the same group had identical exon-intron patterns. Exon counts varied from 1 to 51, and intron counts from 0 to 50. Feng et al. (2021) reported a similar gene structure pattern in cotton, where the exons counts ranged from 1 to 51 (Feng et al., 2021). Results revealed that genes within same group had a similar structure. These results are consistent with findings in cotton (Feng et al.,

2021) and *Brassica napus* (Liu et al., 2018), demonstrating that similarities in the structure of genes and motifs organization were found in the same class genes. Further, *GmCalS* genes functions in the response to environmental stress was revealed by the prediction of *cis*-elements in their promoters. We focus on three distinct *cis*-elements classes: phytohormones, abiotic stress, and plant growth and development-responsive elements. Furthermore, abiotic and phytohormonal stresses are regulated by *cis*-elements in the *GmCalS* genes. Previous research had uncovered *cis*-regulatory elements associated with abiotic stresses and phytohormones. The promoters of *GmCalSs* were discovered to have many hormone-responsive elements, suggesting their involvement in *GmCalSs* regulation. One of the plant's signal molecules was salicylic acid (SA) (Loake and Grant, 2007) increased the expression of *AtCalS1/5/9/10/12* in *Arabidopsis thaliana*. The hormone abscisic acid (ABA), involved in callose synthesis, was crucial in responding to multiple stresses (Liu et al., 2017). Furthermore, ABA biosynthesis up-regulated *PtCalS1* expression and blocked plasmodesmata to maintain the dormant state of *Populus tomentosa* (Tylewicz et al., 2018). Adding methyl jasmonate (MeJA) induced callose deposition in grape leaves. Callose deposition was sped up when *Cationic peroxidase 3* (*OCP3*) expression was suppressed (Repka et al., 2004). *OCP3* functions negatively on the JA pathway. In conclusion, callose deposition was governed by ABA, JA, and SA. However, the presence of ABA, SA, and JA-responsive elements in *GmCalSs* promoters suggests that these hormones regulate the expression of *CalSs* in soybean. The effects of salt stress treatment were also evaluated by analyzing the expression of *GmCalS* genes. Higher expression was observed for a few genes against salt stresses. The previous studies reported that *CalS* genes' expression was increased in response to stress. The *GhCalS3* gene in cotton was up-regulated in response to cold, NaCl, and polyethylene glycol stress (Feng et al., 2021). *CalS1* and *CalS8* are significant genes in *Arabidopsis* that regulate biotic and abiotic stress responses (Cui and Lee, 2016). These findings indicate that *CalS* genes significantly influence plant hormone signaling pathways and abiotic stress tolerance. Plants' ability to cope with stress is directly affected by miRNAs (Villanueva et al., 2016). According to the results of this study, the identified mRNAs target *GmCalS* genes belonging to several families. Similarly, miR156 functions under various abiotic stress conditions in numerous plant species were reported (Arshad et al., 2017; Cui et al., 2016). miR167 was discovered as a key factor in coping with a diverse variety of stimuli (Khraiwesh et al., 2012). In grapevine, miRNA159 expression patterns were discovered. The findings revealed that miRNA159 was participated in gibberellin-induced parthenocarpy (Wang et al., 2018). According to Li et al. (2016), gma-miR172 overexpression in *A. thaliana* displays enhanced tolerance against drought and salt (Li et al., 2016). Also, miRNA-target genes expression validation is important to understand their function in soybean. This study analyzed the expression of 24 *GmCalS* genes in nodules, flowers, leaves, and roots using RNA-seq data. However, the results indicate that *CalS* genes exhibit distinct expression patterns in different developmental tissues. Tissue-specific expression patterns in *Brassica napus* were studied by using qRT-PCR data. According to the findings, *BnCalS* genes had elevated expression in the bud, silique, flower, leaf, stem, and root (Liu et al., 2018). Transcriptome-based expression results revealed that *GhCalS* genes had higher expression in various tissues (Feng et al., 2021). Researchers examined tissue-specific expression in *Pyrus bretschneideri* and found that the *PbrCalS5* gene had higher expression in the pollen tube of pear (Cao et al., 2022). These findings are consistent with the findings of present study, where *CalS* genes displayed higher expression in the examined tissues (nodules, flowers, leaves, and roots), indicating that *CalS* may have a significant role in the development of soybean.

Conclusion

We identified 24 *CalS* genes in the soybean genome. Furthermore, chromosomal location, *cis*-acting elements, conserved motifs, gene structure, and miRNA perdition were analyzed. We discussed the *GmCalS* gene's expression in response to salt stress. However, we find *GmCalS17* and *GmCalS19* genes were enhanced by salt stress. In addition, several *GmCalS* genes were highly expressed in various tissues (roots, leaves, flowers, and nodules). These findings provide a foundation to understand the mechanism of stress resistance in soybean and establish a base for future investigation of the *GmCalS* genes and its function against salt stress.

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Missing "," Review the rules for using punctuation marks.

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Prep. You may be using the wrong preposition.

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Possessive Review the rules for possessive nouns.

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