

Submission date: 22-Nov-2023 12:22PM (UTC-0500) Submission ID: 2236238206 File name: manuscript\_revised\_by\_yasir\_JKSUS\_30-05-2023.doc (328.76K) Word count: 5157 Character count: 30716 1 Genome-Wide Analysis and Expression Profiling of CalS Genes in Glycine max

2 Revealed their Role in Development and Salt Stress

3

### 4 Abstract

Abiotic stress affects plants' growth and development. Soybean is an important crop 5 of the world, however, its production is affected by abiotic stresses. Callose Synthase 6 is the most crucial enzyme response to environmental and developmental signals. 7 However, in soybean, information on the callose synthase genes is limited. In this 8 study, we analyzed the callose synthase gene family of soybean at the genome-wide 9 scale. We also studied the genes positions, gene structure, evolutionary relations, 10 miRNAs target sites, and expression of CalS genes. Resultantly 24 CalS genes were 11 found in soybean, with diverse chromosomal locations, cis-acting elements, conserved 12 13 motifs, and gene structures. Further, GmCalS genes were divided into four phylogenetic classes. The evolutionary classification of *CalSs* was supported by the 14 motif and gene structure analyses. Phytohormones, abiotic stresses, and growth-15 responsive elements were identified in the promoter of GmCalSs. In addition, the 16 GmCalS genes higher expression in roots, leaves, flowers, and nodules tissues 17 provided their significance in development. Furthermore, the higher expression of 18 19 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 20 genes in other crops. 21 22 23 Keywords. Callose Synthase; Expression; Growth; Regulation; qRT-PCR; Salt. 24 25 Introduction Callose is generally found in pollen tubes, grains, cell walls, and root hairs and is 26 essential for transporting intercellular water, cell differentiation, and development 27

28 (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

30 cell to another (Ellinger and Voigt, 2014). Callose Synthase is the most crucial

31 enzyme in callose biosynthesis, with numerous transmembrane segments and a

32 hydrophilic center loop. Furthermore, it responds to environmental and developmental

33 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) 38 examined in Arabidopsis growth retardation was due to AtCalS9, AtCalS10, and 39 40 AtCalS12 genes loss (Barratt et al., 2011). In addition, during the plant's vegetative 41 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 42 43 transport and stops the process of transportation assimilating, leading to the 44 development and growth retardation (Barratt et al., 2011). Shi et al. (2015) 45 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 46 47 (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 48 attack sites (Liu et al., 2018). In addition, Hyaloperonospora arabidopsis and salicylic 49 acid (SA) induce AtCalS1, AtCalS5, AtCalS9, AtCalS10, and AtCalS12 expression 50 (Dong et al., 2008). In *Citrus limon ClCalS1* gene silencing causes more susceptibility 51 to Xanthomonas citri (Enrique et al., 2011). Meanwhile, CalSs are regulated in several 52 signaling pathways. However, hormones and transcription factors participate in 53 different biological regulatory mechanisms. For example, ABA treatment boosts the 54 rice callose synthase activity, and plants resist brown planthopper (BPH) by 55 56 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., 57 2021). 58

The finding of the CalS gene family in many plants revealed their significance in 59 development and response to environmental stress. To date, 15 CalS genes have been 60 identified in Brassica rapa, 7 in Hordeum vulgare, 12 in Zea mays, 8 in Vitis vinifera, 61 32 in Brassica napus, 12 in Citrus sinensis, and 12 in Arabidopsis thaliana (Feng et 62 al., 2021). However, *callose synthase* genes have not been well studied in soybean. 63 Soybeans (*Glycine max*) are important because of their economic and nutritional 64 65 worth. This oil and protein-rich plant contains essential amino acids for humans and 66 other animals. Salt and other environmental stresses pose a danger to soybean 67 production all over the world. Thus, soybean research is crucial for enhancing food

68 security and increasing crop yields. This work investigated chromosomal location,

69 cis-acting elements, conserved motifs, gene structure, and miRNA perdition. In

addition, CalS genes expression was observed in several tissues. Understanding how

71 the *GmCalS* genes respond to salt stress is a foundation for investigating other *CalS* 

72 genes in salt-affected crops.

73 Materials and Methods

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74 Identification of CalS Genes

75 Soybean (Glycine max Wm82.a2.v1) CalS genes were found using BLASTP and

76 HMM approaches. Soybean genome sequences were obtained from the Soybean

77 Genome Database (Schmutz et al., 2010). However, AtCalS arsino acids were utilized

78 as a query in a **BLASTP** search. The amino acid sequences of AtCalSs were obtained

79 from TAIR (http://www.arabidopsis.org/) (Lamesch et al., 2012). Moreover, the

80 HMMER 3.13 program (El-Gebali et al., 2019) was also used to search CalS genes.

81 The HMM file for the CalS domain (PF02364) was obtained from the Pfam database.

82 A total of 24 GmCalSs were identified in the soybean genome after screening the

83 presence of the PF02364 domain in sequences. Data for the *M*. truncatula genome

- 84 was downloaded from the Phytozome JGI 12.0 dataset
- 85 (http://phytozome.jgi.doe.gov/pz/portal.html).

86 Physicochemical Characteristics and Subcellular Localization

87 We predicted the physicochemical characteristics of the GmCalS protein using the

88 ProtParam program (<u>https://web.expasy.org/protparam/</u>) (Gasteiger et al., 2005).

89 CELLO (http://cello.life.nctu.edu.tw/) version 2.5 was used to estimate the subcellular

90 localization of GmCalS proteins. Using the TBtools program, the figure of the exons-

91 introns configuration of GmCalSs was created. The MEME website was used to

92 identify the conserved motifs of GmCalS sequences. The TBtools program was used

93 to construct the motifs distribution.

94 Genes Location and Phylogenetic Analysis

95 The soybean genome generic feature format (GFF) file was used to determine the

96 chromosomal position of the *GmCalSs*. The genes chromosomal locations were

97 determined with the use of TBTools. To better understand the evolutionary

98 relationships among *CalSs*, a phylogenetic tree was built using *AtCalSs*, *MdCalSs*,

99 and *GmCalSs* from the three different plant species. Multiple sequence alignment was

100 carried out using the MEGAX program (Kumar et al., 2018). In this analysis, the

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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 $r = 6.161029 \times 10^9$ ) (Lynch and Conery, 2000).		
Prediction of <i>Cis</i> -Regulatory Elements		
The 2 kb sequences upstream of the start codons in the soybean genome were used to		
determine the <i>cis</i> -regulatory elements in the <i>GmCalS</i> genes promoters. PlantCARE website (Lescot, 2002) was used to analyze the promoter sequences of all <i>GmCalS</i>		
genes, and TBtools software was used to generate the figure.		
Prediction of miRNAs		
To predict miRNAs taget sites, the complementary DNA sequences (CDS) of all		
<i>GmCalSs</i> were submitted to the psRNATarget website		
(https://www.zhaolab.org/psRNATarget/analysis?function=2) (Dai et al., 2018).		
Expression Profiling of <i>GmCalS</i> Genes		
A publicly accessible database was used for the <i>GmCalSs</i> 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 <sup>TM</sup> RT Reagent Kit (TaKaRa, Shiga, Japan) was utilized for the		
cDNA synthesis using $\frac{3}{\mu}g$ 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.

### 137 Results

### 138 Identification and Characterization of *GmCalSs*

To identify genes belonging to the *CalS* family in soybean, **BLASTP** and HMM 139 140 approaches were performed. Twelve AtCalS proteins were used as queries for the 141 BLASTP search. Consequently, 24 GmCalS genes containing the CalS domain with Pfam ID PF02364 were found. These genes were referred to as GmCalS1-GmCalS24. 142 Table 1 provides information on all 24 GmCalS genes. GmCalS genes varied in 143 length as the number of amino acids was from 813 (GmCalS23) to 1965 (GmCalS19). 144 145 The number of exons was from one (GmCalS5 and GmCalS17) to fifty-one 146 (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 147 (GmCalS5 and GmCalS17) (Table 1). The 24 GmCalS proteins were predicted with 148 molecular weights ranging from 92.76 kDa (GmCalS23) to 227.93 kDa (GmCalS22), 149 150 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 151 152 the chloroplast, and 15 GmCalS were present in the nucleus (Table 1).

### 153 CalS Genes Phylogenetic Relationships

154 Here, we construct a phylogenetic tree to understand the evolutionary links between

155 the AtCalS, MtCalS, and GmCalS genes. A. thaliana contains 12 CalS genes and M.

156 truncatula has 15 CalS genes (Supplementary Table S2). The M. truncatula, A.

157 thaliana, and G. max CalS protein sequences were aligned to generate an unrooted

158 phylogenetic tree. The 51 *CalSs* genes from the three plant species were divided into

159 four groups (Figure 1). The 19 Calss in Group A were as follows: 7 from A. thaliana,

160 5 from M. truncatula, K from G. max. Group B included 7 G. max CalSs, 4 M. Sp.

161 truncatula ColSs, NA. thaliana ColS. However, 1 CalSs was found in A. thaliana, Sin

162 M. truncatula, and 5 in G. max in Group C. Furthermore, 3 CalSs were found in A.

163 thaliana, Sin M. truncatula, and 5 in G. max in Group D. CalSs in the same group

164 may perform similar functions. Significantly, *GmCalS* genes showed consistent

distribution across all groups. Group A and B had the highest number of *GmCalSs* (7),

166 followed by C and D (5). It was also discovered that the *GmCalSs* have the strongest

167 evolutionary ties to the *M*. *truncatula* species.

168 Gene Structures and Conserved Motifs Analysis

169 To understand the evolution of the soybean <u>CalSs</u>, we examined the <u>GmCalSs</u> exonintron structures. According to the findings, introns were between 0 and 50 and exons 170 171 from 1 and 51. Overall, there are two genes with a single exon and no intron; three 172 genes with two exons and a single intron; one gene with thirteen fourteen exons; one 173 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 174 175 with forty-five exons and forty-four introns; one gene with fifty exons and forty-nine 176 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, 177 178 consistent with the phylogenetic groups to which they belonged. In addition, the 179 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 180 research, and information on these motifs can be found in Supplementary Table S3. 181 Similar patterns of motif distribution were also seen within the group. Motifs 1, 2, 3, 4, 182 5, 8, and 9 were found in the GmCalS6 gene, whereas motifs 4, 8, 6, and 9 were 183 184 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). 185 186 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, 187 188 GmCalS5, GmCalS7, GmCalS8, GmCalS9, GmCalS10, GmCalS11, GmCalS12, GmCalS15, GmCalS16 GmCalS17, GmCalS18, GmCalS19, GmCalS20, GmCalS21, 189 GmCalS22, GmCalS24 genes. 190

### 191 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 Taks between the *CalS* genes of soybean and *A. thaliana* (Figure 4).

203 Ka/Ks Calculation

204 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 205 selection was occurring among the duplicated genes; when it was less than 1, it was 206 supposed that neutral selection was occurring; and when it was equal to 1, it was 207 assumed that positive selection was occurring (Zaynab et al., 2021). Our results show 208 that purifying selection was applied to most GmCals suplicated genes during 209 duplication. If the Ks values of *GmCalS* genes are higher than 0.52, the deviation time 210 may be more than 100 million years ago (MYA). More intriguingly, the Ks value for 211 the duplicated gene pair (GmCalS5/GmCalS13) was 0.632, indicating that the 212 duplication event happened at about 51.31 MYA (Table 2). 213

214 **Prediction of miRNAs** 

We discovered miRNAs targeting GmCalS genes to understand the miRNA-arbitrated 215 post-transcriptional modification of GmCalSs. These miRNAs are part of different 216 families. Supplementary Table S4 has the data of all miRNA-targeted sites/genes. 217 218 According to the findings, gma-miR159 targeted a total of seven genes (GmCalS1, GmCalS4, GmCalS12, GmCalS14, GmCalS17, GmCalS20, and GmCalS24). The 219 microRNAs, gma-miR172 targeted five genes GmCalS12, GmCalS16, GmCalS19, 220 221 GmCalS20 and GmCalS21 genes; gma-miR171 targeted GmCalS1, GmCalS3, 222 GmCalS4, GmCalS5, GmCalS7, GmCalS9, GmCalS10, GmCalS11,GmCalS14, GmCalS16, GmCalS17, GmCalS19, GmCalS20, and GmCalS21 genes; gma-miR395 223 224 targeted GmCalS11, and GmCalS22 genes; gma-miR394 targeted GmCalS7, 225 GmCalS11, and GmCalS18 genes; gma-miR167 targeted GmCalS3, and GmCalS8 226 genes; gma-miR169 targeted GmCalS2, GmCalS3 GmCalS4, GmCalS7, GmCalS8, GmCalS11, GmCalS12, GmCalS14, GmCalS16, GmCalS17, GmCalS19, GmCalS21, 227 228 *GmCalS22*, and *GmCalS24* genes. It has been discovered that several common genes, such as GmCalS1, GmCalS10, GmCalS12, GmCalS14, and GmCalS20, are targeted 229 230 by mostly different miRNAs

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#### 231 Promoter Analysis of GmCalS Genes

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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

239 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

241 (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

243 inconsistently. In addition, we identified four elements associated with development,

244 including meristem expression, endosperm expression, circadian regulation and zein

245 metabolism. These elements include the O2-site, circadian, CAT-box, and GCN4

246 motif, which perform a dynamic role in the various phases of growth and

247 development of soybean. It is possible to conclude that differential gene expression

248 for *GmCalSs* may occur during different phases of development, and abiotic stress. Sp. @

### 249 *GmCals* Genes Expression in Various Tissues

250 This study utilized RNA-seq data to explore the GmCalS genes expression in flowers. 251 leaves, nodules and roots. The findings demonstrated that several genes had higher 252 expression in several tissues (Figure 6; Supplementary Table S6). The results revealed 253 that in leaves some genes including GmCalS5, GmCalS7, GmCalS9, GmCalS10, 254 GmCalS11, GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS17, GmCalS18, 255 GmCalS19, GmCalS21, and GmCalS24 display higher expression. However, 256 GmCalS3, GmCalS5, GmCalS7, GmCalS8, GmCalS9, GmCalS10, GmCalS11, 257 GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS16, GmCalS17, GmCalS18, GmCalS19, GmCalS21, and GmCalS24 genes display higher expression in roots. In 258 259 nodule, GmCalS5, GmCalS7, GmCalS8, GmCalS9, GmCalS10, GmCalS11, 260 GmCalS12, GmCalS13, GmCalS14, GmCalS15, GmCalS16, GmCalS17, GmCalS18,

261 GmCalS19, GmCalS21 and GmCalS24 genes display higher expression. Several

262 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.

## 268 *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.

275 Discussion

Plants have contact with their surroundings therefore subjected to abiotic and biotic 276 277 stresses. Abiotic stress factors affect plants' anatomy, physiology, biochemistry, and 278 morphology, significantly reducing their growth and development (Nadarajah, 2020). 279 Several studies have reported the function of callose in development of plant and 280 against stresses (Piršelová and Matušíková, 2013; Verma and Hong, 2001). Due to 281 callose significance, *callose synthase* has been studied in several plants. Eight *Callos* were identified from Vitis vinifera, 15 CalSs from Chinese cabbage, 12 CalSs from 282 283 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. 284 285 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 286 287 genes, which is higher than the number of CalS genes in Arabidopsis. This is evidence 288 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 289 patterns. Exon counts varied from 1 to 51, and intron counts from 0 to 50. Feng et al. 290 291 (2021) reported a similar gene structure pattern in cotton, where the exons counts 292 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., 293

294 2021) and *Brassica napus* (Liu et al., 2018), demonstrating that similarities in the 295 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 296 prediction of cis-elements in their promoters. We focus on three distinct cis-elements 297 classes: phytohormones, abiotic stress, and/plant growth and development-responsive 298 299 elements. Furthermore, abiotic and phylohormonal stresses are regulated by *cis*elements in the GmCalS genes. Previous research had uncovered cis-regulatory 300 301 elements associated with abiotic stresses and phytohormones. The promoters of GmCalSs were discovered to have many hormone-responsive elements, suggesting 302 their involvement in GmCalSs regulation. One of the plant's signal molecules was 303 304 salicylic acid (SA) (Loake and Grant, 2007) increased the expression of 305 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., 306 307 2017). Furthermore, ABA bios Inthesis up-regulated *PtCalS1* expression and blocked plasmodesmata to maintain the dormant state of *Populus tomentosa* (Tylewicz et al., 308 309 2018). Adding methyl jasmonate (MeJA) induced callose deposition in grape leaves. Callose deposition was sped up when Cationic peroxidase 3 (OCP3) expression was 310 suppressed (Repka et al., 2004). OCP3 functions negatively on the JA pathway. In 311 312 conclusion, callose deposition was governed by ABA, JA, and SA. However, the presence of ABA, SA, and JA-responsive elements in *GmCalSs* promoters suggests 313 314 that these hormones regulate the expression of *CalSs* in soybean. The effects of salt 315 stress treatment were also evaluated by analyzing the expression of GmCalS genes. 316 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 317 318 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 319 that regulate blotic and abiotic stress responses (Cui and Lee, 2016). These findings 320 321 indicate that  $\mathcal{L}alS$  genes significantly influence plant hormone signaling pathways and abiotic streams tolerance. Plants' ability to cope with stress is directly affected by 322 323 miRNAs (Villanueva et al., 2016). According to the results of this study, the identified mRNAs target GmCalS genes belonging to several families. Similarly, 324 325 miR156/ functions under various abiotic stress conditions in numerous plant species 326 were reported (Arshad et al., 2017; Cui et al., 2016). miR167 was discovered as a key 327 factor in coping with a diverse variety of stimuli (Khraiwesh et al., 2012). In

grapevine, miRNA159 expression patterns were discovered. The findings revealed 328 that miRNA159 was participated in gibberellin-induced parthenocarpy (Wang et al., 329 2018). According to Li et al. (2016), gma-miR172 overexpression in A. thaliana 330 331 displays enhanced tolerance against drought and salt (Li et al., 2016). Also, miRNAtarget genes expression validation is important to understand their function in soybean. 332 333 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 334 335 distinct expression patterns in different developmental tissues. Tissue-specific expression patterns in Brassica napus were studied by using qRT-PCR data. 336 According to the findings, BnCalS genes had elevated expression in the bud, silique, 337 338 flower, leaf, stem, and root (Liu et al., 2018). Transcriptome-based expression results 339 revealed that GhCalS genes had higher expression in various tissues (Feng et al., 2021). Researchers examined tissue-specific expression in Pyrus bretschneideri and 340 341 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 342 343 *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 344 345 of soybean.

#### 346 Conclusion

We identified 24 CalS genes in the soybean genome. Furthermore, chromosomal 347 location, cis-acting elements, conserved motifs, gene structure, and miRNA perdition 348 were analyzed. We discussed the GmCalS gene's expression in response to salt 349 stress. However, we find GmCalS17 and GmCalS19 genes were enhanced by salt 350 351 stress. In addition, several *GmCals* genes were highly expressed in various tissues (roots, leaves, flowers, and nodules). These findings provide a foundation to 352 353 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. 354 Funding 355

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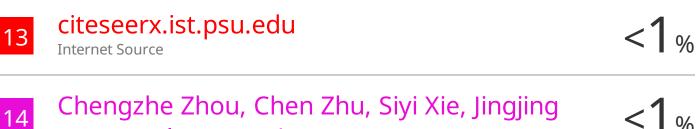
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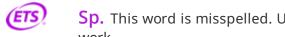
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PAGE 7



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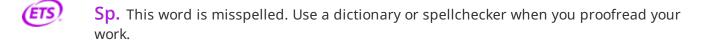
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**ETS**) **Proper Nouns** You may need to use a capital letter for this proper noun.

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- **Proper Nouns** You may need to use a capital letter for this proper noun.
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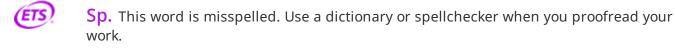


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**P/V** You have used the passive voice in this sentence. You may want to revise it using the active voice.

PAGE 8

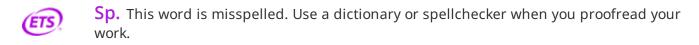


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**Missing** "," Review the rules for using punctuation marks.

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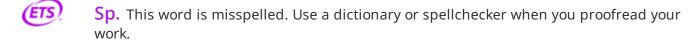
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Article Error You may need to use an article before this word. Consider using the article the.



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

Wrong Article You may have used the wrong article or pronoun. Proofread the sentence to make sure that the article or pronoun agrees with the word it describes.

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Article Error You may need to use an article before this word.

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**Proofread** This part of the sentence contains an error or misspelling that makes your meaning unclear.



**Proofread** This part of the sentence contains an error or misspelling that makes your meaning unclear.

Wrong Article You may have used the wrong article or pronoun. Proofread the sentence to make sure that the article or pronoun agrees with the word it describes.



**Prep.** You may be using the wrong preposition.





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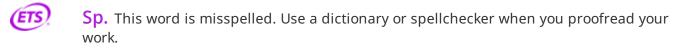


**Article Error** You may need to use an article before this word. Consider using the article **the**.



**Article Error** You may need to use an article before this word. Consider using the article **the**.

### PAGE 10



- **Proofread** This part of the sentence contains an error or misspelling that makes your meaning unclear.
  - Article Error You may need to remove this article.



**Possessive** Review the rules for possessive nouns.



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