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ARTICLE

# Genome-Wide Identification and Expression Analysis of the *GSK3* Gene Family in Sunflower under Various Abiotic Stresses

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## ABSTRACT

Genes in the glycogen synthase kinase 3 (*GSK3*) family are essential in regulating plant response to stressful conditions. This study employed bioinformatics to uncover the *GSK3* gene family from the sunflower genome database. The expressions of *GSK3* genes in different tissues and stress treatments, such as salt, drought, and cold, were assessed using transcriptome sequencing and quantitative real-time PCR (qRT-PCR). The study results revealed that the 12 *GSK3* genes of sunflower, belonging to four classes (Classes I–IV), contained the GSK3 kinase domain and 11–13 exons. The majority of *GSK3* genes were highly expressed in the leaf axil and flower, while their expression levels were relatively lower in the leaf. As a result of salt stress, six of the *GSK3* genes (*HaSK11*, *HaSK22*, *HaSK23*, *HaSK32*, *HaSK33*, and *HaSK41*) displayed a notable increase in expression, while HaSK14 and HaSK21 experienced a significant decrease. With regard to drought stress, five of the *GSK3* genes (*HaSK11*, *HaSK13*, *HaSK21*, *HaSK22*, and *HaSK33*) experienced a remarkable rise in expression. When exposed to cold stress, seven of the *GSK3* genes (*HaSK11*, *HaSK12*, *HaSK13*, *HaSK32*, *HaSK33*, *HaSK41*, and *HaSK42*) showed a substantial increase, whereas *HaSK21* and *HaSK23* had a sharp decline. This research is of great importance in understanding the abiotic resistance mechanism of sunflowers and developing new varieties with improved stress resistance.

## KEYWORDS

Sunflower; abiotic stress; GSK; expression analysis

## 1 Introduction

Glycogen synthase kinase 3 (GSK3) is a conserved serine/threonine protein kinase, and is also referred to as SHAGGY-like protein kinase. GSK3 was initially found in rabbit skeletal muscle, and existed in two isoforms (GSK3α and GSK3β) in mammals [1]. Subsequently, GSK3 proteins are identified in various plants, including 10 in Arabidopsis, nine in rice, and nine in potatoes [2,3]. These discoveries suggest a greater diversity of the *GSK3* gene family in plants than the corresponding genes in animals.

GSK3 protein contributes to the formation of flowers, stomata, seeds, plant height, flowering time and root systems in plants. BRASSINOSTEROID INSENSITIVE2 (BIN2), a GSK3-like kinase, has been identified to phosphorylate the transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and Brassinazole resistant 1 (BZR1), thus inhibiting the signal transduction pathway of brassinolide (BR) and

regulating plant growth [4,5]. Research has revealed that BIN2 and BIL1/2 can mediate root growth and seed germination by antagonizing BR and abscisic acid (ABA) pathways [6–8]. Claisse et al. observed that the overexpression of *AtSK32-R178A* resulted in shorter pedicels and smaller petals than the wild-type controls [9]. GSK3 phosphorylated and regulated Rht-B1b to reduce plant height in wheat [10]. GSK3 could interact with VRN1 and regulate its accumulation to mediate flowering in wheat [11]. GSK3-like kinase ZmSK2, a maize homolog of BIN2 in Arabidopsis, was proven to be involved in embryonic development [12].

GSK3 has additionally been linked to responses to biotic and abiotic stressors [3,13]. Research has demonstrated that *AtSK13*, *AtSK31*, and *AtSK42* are all activated in response to salt stress [14]. The overexpression of *AtSK22* has been associated with improved salt tolerance in plants [15]. Evidence from the research of Dal Santo et al. [16] and Stampfl et al. [17] suggested that increased expression of *AtSK11* augmented salt tolerance in plants and decreased their sensitivity to *Pseudomonas syringae*. Moreover, Yang et al. [18] found that increased expression of *OsGSK3* in rice enhanced ABA responsiveness as well as tolerance to salt, cold, drought, and mechanical damage. AtBIN2 has been demonstrated to interact with RD26 and augment the drought tolerance of plants [19]. AtBIN2 can also phosphorylate and impede the activity of SOS2, thereby inhibiting the salt stress tolerance of Arabidopsis [20]. Under cold stress, BIN2 can phosphorylate and bolster the activity of BZR1, CESTA, and ICE1, thereby improving the cold tolerance of seedlings [21–23] and raising the level of C-REPEAT BINDING FACTOR (CBF). Researchers have discovered that the interaction between BIN2 and JAZ proteins lessens plant resistance to *Verticillium wilt*, as reported by Song et al. [24]. *GmSK2-8*, which can be strongly induced under high-salt conditions, interacted with GmNSP1a and GmNSP1b to mediate salt-inhibited legume-rhizobiumsymbiosis [25].

*Helianthus annuus* L. is an essential oil crop and ornamental plant. However, its production is significantly influenced by numerous abiotic and biotic stresses. Although the *GSK3* gene family is implicated in many stress response processes of plants, there is limited information about the *GSK3* gene family in sunflowers. The present investigation aimed to identify the *GSK3* genes of sunflowers and analyze their protein sequence, gene structure, *cis*-acting elements, and expression traits across diverse tissues and treatments. This research serves as a fundamental basis for further exploring the role of *GSK3* genes in stress in plants.

## 2 Materials and Methods

### *2.1 Plant Growth Condition*

In this study, *H. annuus var.* 'huoli' was used. The sunflower plants were grown in nutrient-rich soil in square pots and put in a tissue culture room under the following growth conditions: 5000 lx, 16/8 h (light/dark), 23 ± 2°C.

### *2.2 Identification of GSK3 Genes in Sunflower*

The protein sequences in the sunflower genome database (https://www.heliagene.org/HanXRQr2.0-SUNRISE/, accessed on 20 May 2024) were screened using the sunflower GSKs as queries. After removing the repetitive sequences and those without the GSK3 domain, the *GSK3* genes of the sunflower were determined. To validate sunflower GSKs, the Hidden Markov Model (HMM) was employed. To get the molecular weights, theoretical isoelectric points, and number of amino acids in each sunflower GSK sequence, the sequences were uploaded to ExPASy (http://web.expasy.org/protparam/, accessed on 20 May 2024) for analysis. The sequences of Arabidopsis GSKs were obtained from NCBI.

### *2.3 Phylogenetic, Gene Structure, and Motif Distribution*

The MEME Suite 5.0.5 was utilized to examine the conservative motifs of sunflower *GSK3* genes. To validate the *GSK3* gene structure, GSDS 2.0 software (http://gsds.cbi.pku.edu.cn/, accessed on 20 May 2024)

was employed. To construct a maximum likelihood phylogenetic tree of the GSK3 protein sequence of sunflower and Arabidopsis, Clustal X 1.8 and MEGA 11.0 were employed and bootstrap repeat analysis was conducted 1000 times [26].

### *2.4 Prediction of the Cis-Regulatory Elements in GSK3 Genes*

The upstream flanking sequence (2000 bp) from the transcription start site of each putative sunflower *GSK3* gene was downloaded. Utilizing PlantCARE online software, the conserved *cis*-elements in the promoter regions of sunflower *GSK3* genes were studied.

### *2.5 Expression Profile Analysis of GSK3 Genes*

RNA was extracted from the buds, roots, leaves, stems, and flowers of *H. annuus* var. 'huoli'. RNA extraction, RNA sequencing, and data analysis were all conducted according to the method described by Dong et al. [27].

*H. annuus* var. 'huoli' seeds were grown in pots with mixed soil (1:1, vermiculite/humus) for a duration of 30 days. Then the seedlings were irrigated with either 300 mM NaCl or 20% PEG 6000. The control group was irrigated with water. Six hours later, the sunflower leaves were harvested for gene expression analysis [28]. The 30-day old seedlings were placed in an incubator at 5°C for cold treatment, while the control group was subjected to 25°C. Six hours later, the leaves were gathered.

Total RNA extraction was performed with TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China) following the manufacturer's protocol. Utilizing MonScript™ RTIII all-in-one mix with dsDNase (Monad, Wuhan, China), the first cDNA strand was generated from 1 μg of total RNA. Using the SYBR Green PCR Master Mix (Toyobo, Shanghai, China), qRT-PCR assay was conducted on an ABI 7500 Fast real-time PCR system. The reaction procedure was as follows: denature at 95°C for 1 min, followed by 45 cycles of 95°C for 20 s, 50°C–60°C for 20 s, 72°C for 30 s.

To obtain accurate results, three biological and technical replicates were employed for every treatment. For each biological replicate, five sunflower plants were utilized. *Elongation factor 1 alpha* (*EF1α*) was employed as the reference gene (Table S1). Relative gene expression were calculated based on the $2^{-\Delta\Delta Ct}$ method [29].

### *2.6 Statistical Analysis*

Data was reported as mean value ± standard deviation (SD). Student's *t*-tests were conducted for statistical analyses.

## 3 Results

### *3.1 Identification and Classification of GSK3 Gene Family in Sunflower*

We analyzed the sunflower genomic database and identified 12 *GSK3* genes. The proteins encoded by the *GSK3* genes displayed a range of lengths from 381 amino acids (HaSK22) to 516 amino acids (HaSK13). Molecular weights ranged from 43.2 kDa (HaSK22) to 59.0 kDa (HaSK13). Isoelectric points from 6.34 (HaSK31) to 9.03 (HaSK32). According to predictions regarding subcellular localization, all GSK3 proteins were present in the nucleus (Table 1).

Sequencing anazlysis showed that the protein sequences of GSK3 members were highly conserved across the kinase domain, while the N- and C-terminal regions are variable. The multiple conserved subdomains of GSK3 proteins included APE, GxGxxG, xAxK, RD, SYICSR, CDFGSAK and the TREE motif (Fig. 1). Among them, the presence of SYICSR and CDFGSAK motifs can distinguish HaSK proteins from other serine/threonine protein kinases, while the TREE motif is a unique feature of plant GSK proteins.

**Table 1:** Basic information of *GSK3* family genes in sunflower

| Gene name | Gene ID | Number of amino acids | Subcellular localization | Molecular weight/kDa | Isoelectric point |
|---|---|---|---|---|---|
| HaSK11 | HannXRQ_Chr06g0168041 | 406 | Nucleus | 46.03 | 8.93 |
| HaSK12 | HannXRQ_Chr09g0266281 | 411 | Nucleus | 46.35 | 8.19 |
| HaSK13 | HannXRQ_Chr14g0450461 | 516 | Nucleus | 58.95 | 7.94 |
| HaSK14 | HannXRQ_Chr07g0198881 | 410 | Nucleus | 46.5 | 8.22 |
| HaSK21 | HannXRQ_Chr11g0341851 | 384 | Nucleus | 43.43 | 8.51 |
| HaSK22 | HannXRQ_Chr04g0115771 | 381 | Nucleus | 43.22 | 8.05 |
| HaSK23 | HannXRQ_Chr15g0480751 | 401 | Nucleus | 45.46 | 6.67 |
| HaSK31 | HannXRQ_Chr13g0426211 | 470 | Nucleus | 53.09 | 6.34 |
| HaSK32 | HannXRQ_Chr06g0169741 | 447 | Nucleus | 50.17 | 9.03 |
| HaSK33 | HannXRQ_Chr04g0121911 | 445 | Nucleus | 50.35 | 8.71 |
| HaSK41 | HannXRQ_Chr03g0067951 | 425 | Nucleus | 48.4 | 7.61 |
| HaSK42 | HannXRQ_Chr10g0299521 | 425 | Nucleus | 48.18 | 8.54 |

### *3.2 Phylogenetic Analysis and Grouping of GSK3 Proteins*

A phylogenetic tree with 10 Arabidopsis GSK3 proteins, nine rice GSK3, and 12 sunflower GSK3 proteins was created to examine (Fig. 2). Using the Arabidopsis and rice GSK3 protein groups as a reference, 12 GSK3 proteins have been divided into four subgroups (I–IV). Group I had four members, including HaSK11, HaSK12, HaSK13, HaSK14. Group II had HaSK21, HaSK22, HaSK23. Group III had HaSK31, HaSK32, and HaSK33. Group IV comprised two members, including HaSK41 and HaSK42.

### *3.3 Analysis of GSK3 Gene Structure in Sunflower*

Analysis of the structure of sunflower *GSK3* genes revealed that all *HaSK* genes contained 11–13 exons, especially all genes in Group IV had 12 exons. Additionally, MEME software identified 13 protein motifs in sunflower GSK3. The majority of GSK3 proteins belonging to the same group shared a motif, indicating that their functions might be comparable. The HaSK proteins all contained motif 1–7 and motif 9, which together make up the GSK3 kinase domain. All proteins of Groups I, II, and IV contained motif 8, whereas members of Groups I (apart from HaSK11), III, and IV primarily contained motif 10. Notably, motif 13 was detected in both Group IV and Group I members (apart from HaSK11 and HaSK13) (Fig. 3).

### *3.4 Analysis of Cis-Acting Elements of GSK3 Gene Promoters in Sunflower*

Potential roles for *HaSK* genes in regulating stress-coping response were identified by analyzing the 2000 bp sequence upstream of the initial codon. Growth and development, stress, hormones, and light response are the four categories into which the *cis*-regulatory elements were separated. Elements associated with growth and development included MSA-like and CAT-box. Stress-related regulation has been linked to drought-responsive elements (MBS) and low-temperature-responsive elements (LTR, WRE3). The hormone-related elements were jasmonic acid-responsive element (TGACG motif, CGTCA motif), gibberellic acid (GA)-responsive element (GARE motif, P-box), auxin-responsive element (TGA-element), salicylic acid-responsive element (TCA-element), and ABA-responsive element (ABRE). The elements that were responsive to light were Sp1, GT1-motif, MRE, CCAAT-box, and G-box (Fig. 4).

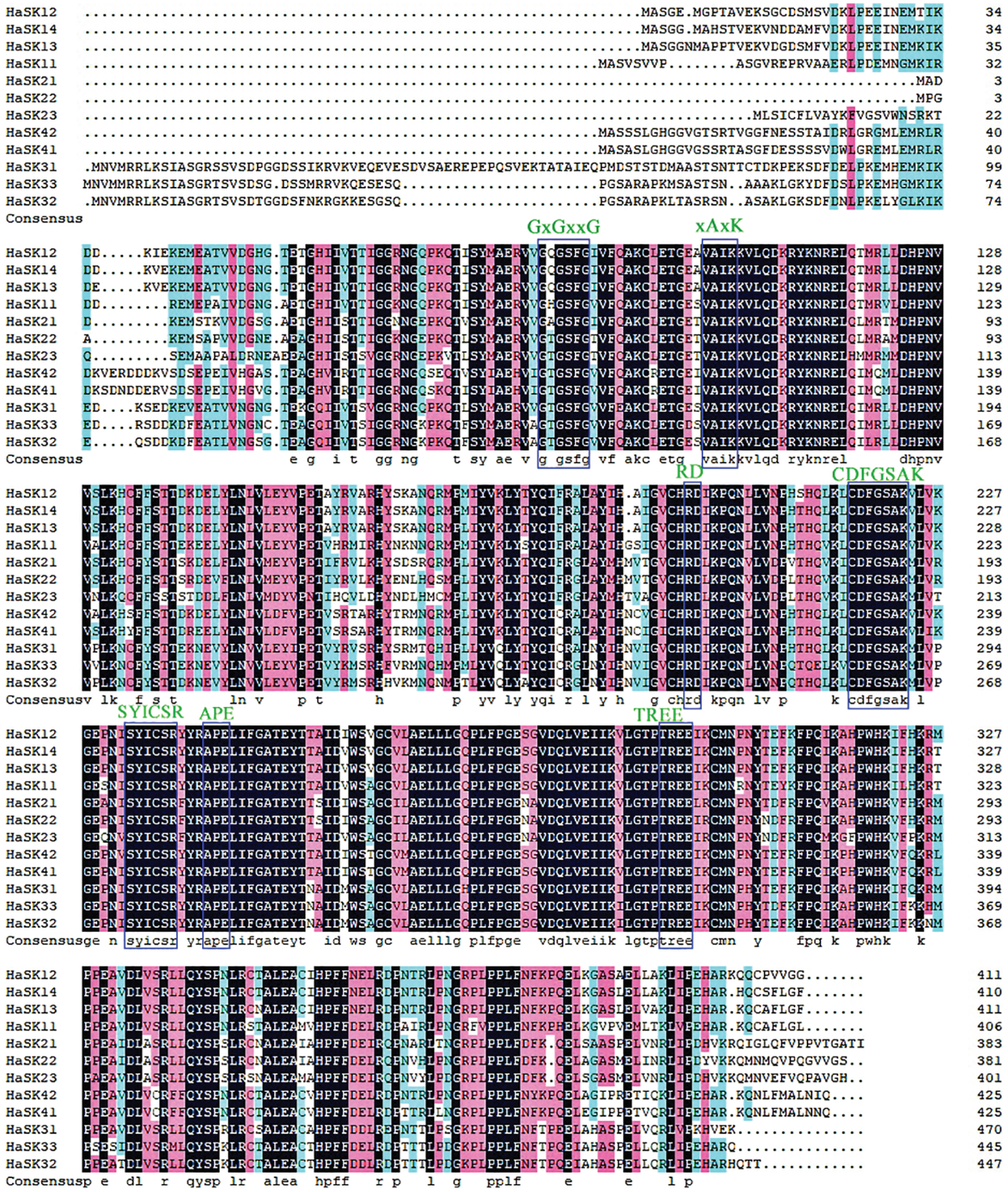


**Figure 1:** Sequence alignment of sunflower GSK3 proteins. Note: The subdomains of kinase domain were marked with blue frames

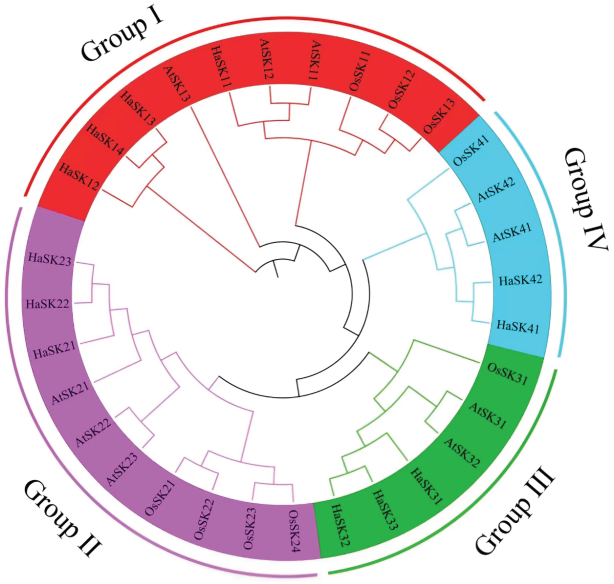


**Figure 2:** Phylogenetic tree of GSK3s from sunflower (Ha), rice (Os) and Arabidopsis (At). The four groups could be identified using different colors

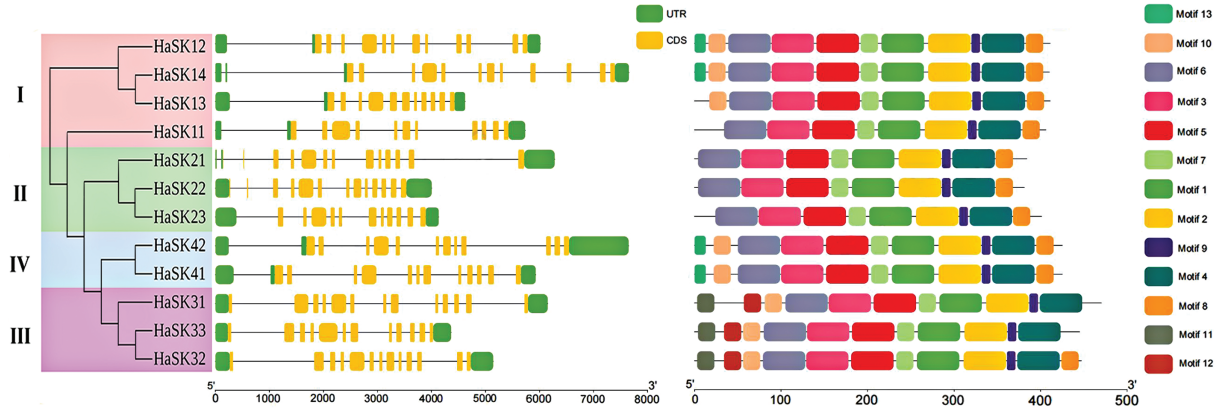


**Figure 3:** Gene structure, motifs, and phylogenetic relationships of sunflower GSK3s. Exons are represented as yellow boxes, introns are represented as black lines, and the green boxes represent the UTR of sunflower *GSK3* genes. The thirteen putative motifs are presented by boxes of different colors

### *3.5 Expression Profiles of GSK3 Genes in Different Tissues of Sunflower*

We conducted transcriptome sequencing to investigate the expression levels of the *GSK3* genes in sunflowers. Our findings revealed that the expressions of the *GSK3* genes were the greatest in flowers (*HaSK22*, *HaSK23*, *HaSK41*, and *HaSK42*) and leaf axils (*HaSK14*, *HaSK32*, *HaSK33*). In contrast, their expression levels in leaves were comparatively lower (Fig. 5). This suggests that *GSK3* genes of sunflowers might be implicated in flower and axillary bud development.

### *3.6 Expression Profile of GSK3 Genes in Sunflower under Salt Stress*

The expression levels of *HaSK11*, *HaSK22*, *HaSK23*, *HaSK32*, *HaSK33* and *HaSK41* were significantly impacted after 6 h of NaCl treatment. The levels of *HaSK22* and *HaSK23* increased by more than ten times.

*HaSK14* and *HaSK21*, on the other hand, showed a marked decline in expression levels (Fig. 6). These results imply that the sunflower's adaptation to salt stress may be mediated by these genes.

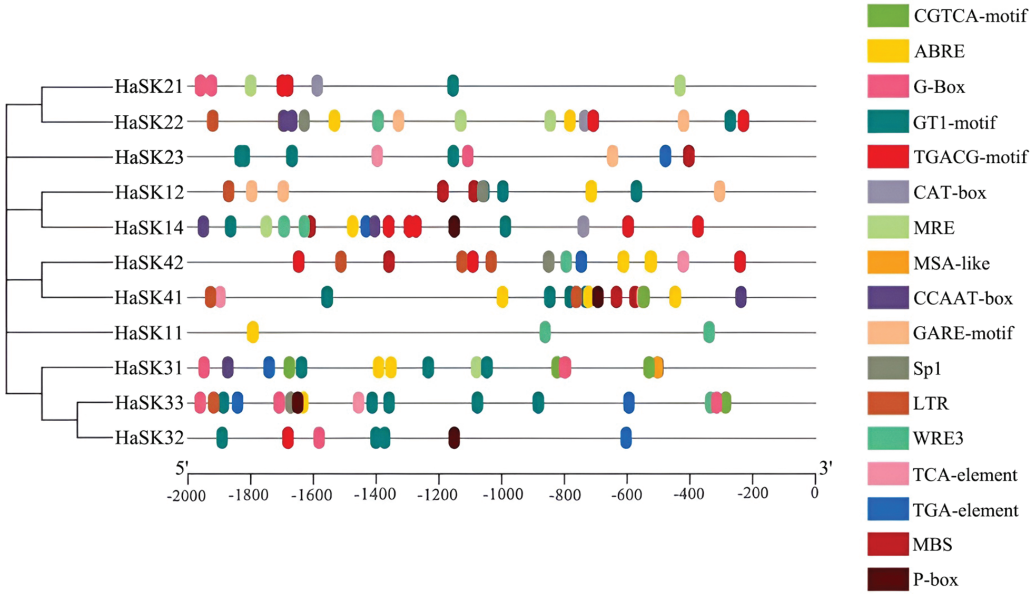


**Figure 4:** Types of *cis*-acting elements in promoter regions of sunflower *GSK3* genes. The bottom scale can be used to determine the upstream length to the translation start site

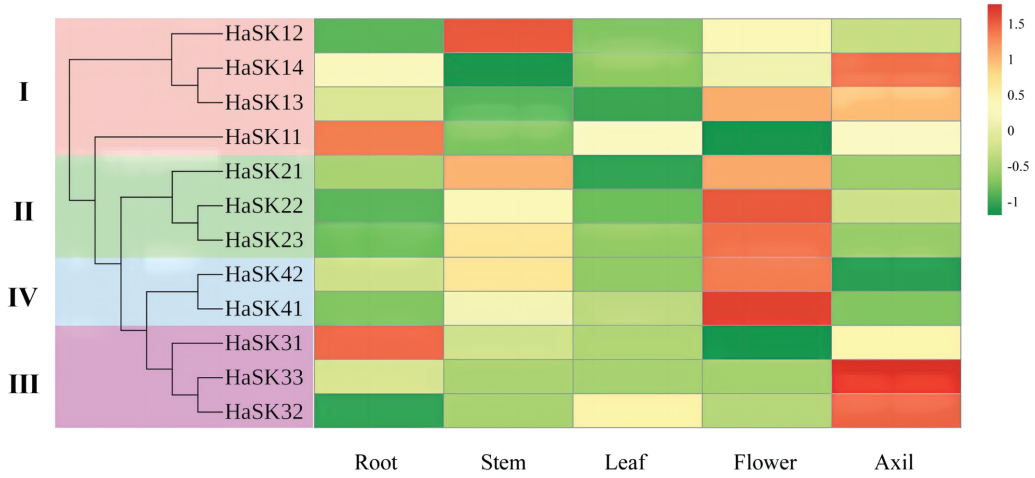


**Figure 5:** Analysis of GSK3 gene expression in five different sunflower tissues. The FPKM values of the HaSK genes were converted using log2

### *3.7 Expression Profile of GSK3 Genes to Drought Stress in Sunflower*

To determine the reaction of *HaSK* genes to drought stress in sunflowers, 20% PEG 6000 solution was applied. The expression levels of *HaSK* genes were determined after 6 h. It was observed that the expression levels of *HaSK11*, *HaSK13*, *HaSK21*, *HaSK22*, and *HaSK33* were significantly elevated (Fig. 7). These findings indicate that these genes might be involved the sunflower's ability to withstand drought conditions.

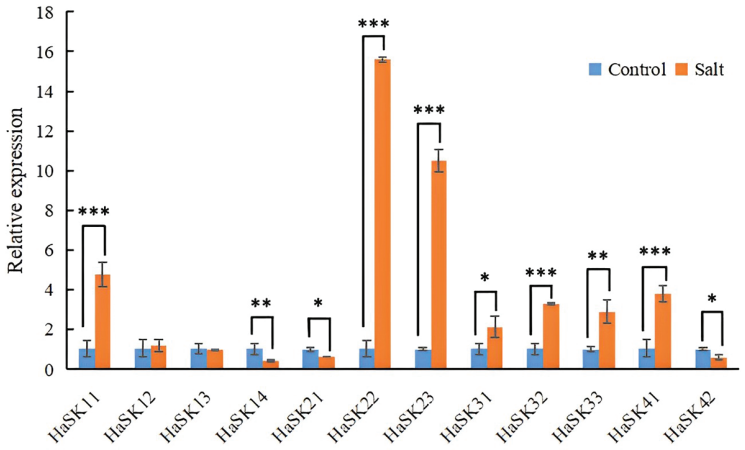


**Figure 6:** Expressions profile of *GSK3* genes in response to NaCl treatment. The results were represented by mean ± SD. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by *t*-test

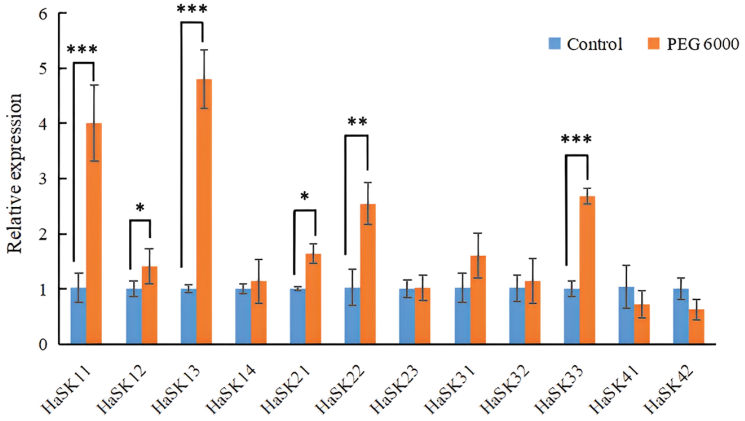


**Figure 7:** The profiles of *GSK3* gene expression in response to drought stress treatment. The results were represented by mean ± SD. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by *t*-test

### *3.8 Expression Profile of GSK3 Genes in Sunflower to Cold Stress*

The expression levels of *HaSK* genes were assessed 6 h after the plants were exposed to a temperature of 5°C to evaluate the effects of cold stress on sunflowers. The results revealed that the expressions of *HaSK11*, *HaSK12*, *HaSK13*, *HaSK32*, *HaSK33*, *HaSK41*, and *HaSK42* were significantly increased. Conversely, the levels of *HaSK21* and *HaSK23* were significantly decreased. These results suggest that these genes might be implicated in the sunflower's reaction to cold stress (Fig. 8).

## 4 Discussion

This study identified 12 *GSK3* genes in sunflowers, more than those found in rice [30], Arabidopsis [31] and potatoes [3]. To further investigate the GSK3 proteins, phylogenetic analysis, exon/intron structure and conservative motif analysis were conducted. Results revealed that GSK3 proteins were divided into four groups, with a similar gene structure within each group (Fig. 3). Additionally, a low degree of similarity between the N- and C-terminal amino acid sequences was found through comparison, indicating a functional divergence among GSK3 members. Additionally, the homology of the kinase domain was found to be high, which was confirmed by the results of the conservative motif analysis (Fig. 1). This

kinase domain consists of multiple conserved subdomains, as previously reported by Bittner et al. [32]. These subdomains are essential for regulating plant growth and maintaining kinase activity. Research by Kim et al. [31] found that a mutation of the lysine (K) to arginine (R) within the xAxK motif decreased kinase activity.

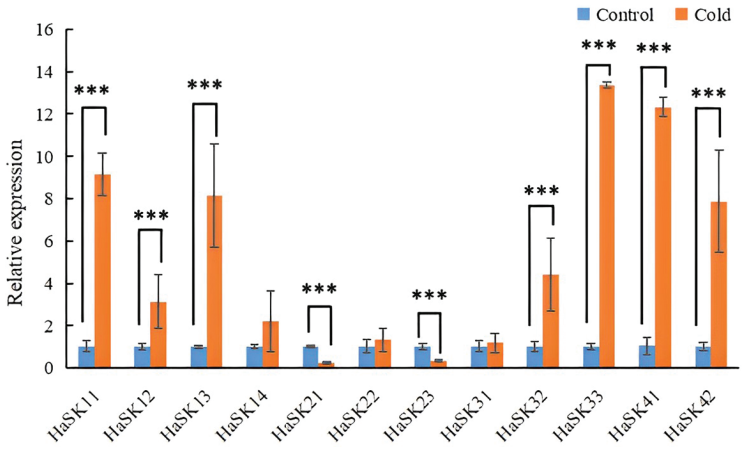


**Figure 8:** *GSK3* gene expression profile in reaction to cold stress. The results were represented by mean ± SD. ***$p < 0.001$ by *t*-test

Tissue-specific expression profile analysis showed that Groups II and IV genes were expressed similarly, with the highest expression in flowers and the lowest expression in leaves, roots, and leaf axils (Fig. 5). These results imply that these genes regulate the development of these tissues in a similar manner. However, even though HaSK11, HaSK12, HaSK13, and HaSK14 were classified as subgroup I, their expression patterns varied. For instance, *HaSK11*, *HaSK12*, and *HaSK14* demonstrated the highest expressions in stems, roots, and leaf axils, respectively (Fig. 5), indicating that there may be functional differences between the genes in the same branch. Together, GSK3 proteins have a multifunctional capacity in plants.

According to an analysis of *cis*-regulatory elements in the gene, several elements linked to growth responses were found in the sunflower *GSK3* gene promoter, stress, hormones, and light, which is similar to findings reported in cotton and potato [3,13]. This implies that the function of this gene family is conserved across different species. ABRE, TGACG motif, and CGTCA motif, associated with ABA and jasmonic acid response, were present in most *GSK3* genes. *GSK3* genes were found to be associated with various elements, such as GA response, auxin response, salicylic acid response, stress, and growth and development, indicating their diverse roles.

Studies in various species have demonstrated that *GSK3* genes, such as *AtSK11*, *AtSK22*, *ASKα*, *TaSK5*, and *GmBIN2*, are implicated in multiple of biological stress responses [13,15,16,33,34]. The analysis of the expression level of sunflower *GSK3* genes under various abiotic stressors found that *HaSK11* and *HaSK33* responded to all three stresses. Most *GSK3* genes only responded to two of them, such as *HaSK13*, *HaSK22*, *HaSK32*, and *HaSK41*. Furthermore, it was found that under abiotic stress, most of the genes' expression levels dramatically increased, and some genes decreased significantly, such as *HaSK14*, *HaSK21*, and *HaSK23*. Notably, the expression level of *HaSK22*, closely related to *GmBIN2* (Fig. S1), was significantly increased in the presence of drought and salt stress.

Transgenic Arabidopsis and peas that overexpress *GmBIN2* have improved resistance to salt and drought stress [13]. This finding suggests that *HaSK22*, known to respond to salt and drought stress, may regulate sunflowers' salt and drought resistance. Phylogenetic tree analysis revealed that OsGSK3 and GmGSK were closely related to HaSK11 (Fig. S1). Research has indicated that *OsGSK3* and *GmGSK* expression

levels can be elevated by salt, drought, and cold stress [18,35]. *HaSK11* expression was detected in sunflowers, indicating that *HaSK11* is critical in regulating the abiotic stress of sunflowers, and its function is relatively consistent across species. Furthermore, phylogenetic tree analysis showed that StSK21 was closely related to HaSK21 (Fig. S1). The overexpression of *StSK21* in Arabidopsis caused a decrease in seed germination rate and increased salt sensitivity of transgenic plants [3]. On the other hand, *HaSK21* expression was significantly reduced under salt stress, which is in line with the findings in potatoes. These findings indicate that *HaSK21* may negatively regulate salt stress tolerance in sunflowers.

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**Availability of Data and Materials:** All of the data developed or examined during this investigation are available in this published article and its supplementary materials, or they can be provided at reasonable request.

**Ethics Approval:** Not applicable.

**Conflicts of Interest:** The authors declare that they do not have any conflicts of interest to disclose in relation to this study.

**Supplementary Materials:** The supplementary material is available online at https://doi.org/10.32604/phyton.2024.052809.

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