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Genome-Wide Identification of the *KCS* Gene Family in Foxtail Millet (*Setaria italica* L.)

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Received: 09 January 2026; Accepted: 24 March 2026; Published: 28 April 2026

ABSTRACT: Very long-chain fatty acids (VLCFAs) are widely distributed across plant tissues. 3-Ketoacyl-CoA synthase (KCS) is one of the most crucial enzymes in VLCFA synthesis and markedly influences fatty acid composition in plants. However, the relevant information on KCS proteins in foxtail millet remains poorly understood. In the current study, 30 *KCS* genes were found in foxtail millet using bioinformatics methods. Phylogenetic data indicated that these genes cluster into eight distinct groups, with members of each group sharing similar motif structures. Further analysis revealed that the *cis*-acting elements of *SiKCS* genes are mainly involved in growth and developmental processes. Synteny analysis indicates that segmental duplications are crucial for the expansion of the *KCS* genes. Expression profiles results indicated that 22 *SiKCS*s were highly expressed in the foxtail millet seed. This comprehensive analysis provided important insights for the functions of *KCS* genes and identified potential genetic resources for precision breeding enhancement in foxtail millet.

KEYWORDS: Foxtail millet; very long-chain fatty acid; *KCS* gene family; genome-wide analysis

1 Introduction

Foxtail millet (*Setaria italica* L.) originated in China and historically served as a primary food crop [1,2]. This species possesses several agronomic advantages, including short growth cycles, robust resistance to abiotic stress, and low requirements for water and fertilizer [3,4]. In addition, dehusked foxtail millet offers unique nutritional advantages, providing essential starch, proteins, and vitamins. Moreover, it has a high dietary fiber content, rendering it highly beneficial for the digestive system [5]. Foxtail millet also contains elevated concentrations of phenols and carotenoids, which possess significant antioxidant and antibacterial properties [6]. In addition to providing abundant nutrients, the grain contains a variety of fatty acids, which are vital for promoting human health and preventing disease [7–9].

Very long-chain fatty acids (VLCFAs) are long-chain fatty acids consisting of 18 or more carbon atoms. These molecules can be derivatized, modified, esterified to other molecules, or polymerized to create physiologically active compounds [10]. In eukaryotes, the elongation of VLCFAs occurs primarily in the endoplasmic reticulum through fatty acid elongation (FAE) complexes. This process is catalyzed by 3-ketoacyl-CoA synthase (KCS) and other major enzymes [11].

KCS is the primary rate-limiting enzyme in FAE, affecting the chain length and yield of VLCFAs. This enzyme catalyzes the initial step of fatty acid chain extension. Different KCS isoforms exhibit substrate specificity for specific chain lengths. Their activities directly control the rates of VLCFA synthesis and

serve as key regulatory points for the synthesis of waxes, cuticular layers, and seed oils in plants, as well as sphingolipids in mammals [12–16]. Given these specialized features, numerous *KCS* genes have been identified and characterized. These genes are recognized for their significant influence on the regulation of plant fatty acid composition. For example, specific amino acid substitutions in the product of the *KCS18* protein affect fatty acid profiles in *Arabidopsis* [17]. Furthermore, *AhKCS* gene editing reduces saturated fatty acid content in peanut, thereby enhancing its nutritional quality [18–20]. The overexpression of *KCS* genes has also been shown to increase nervonic acid content in various plant seeds [21,22]. Additionally, *AKR2A* interacts with *KCS1*, consequently modulating VLCFA biosynthesis in cotton [23].

At present, a systematic investigation of *KCS* genes in foxtail millet is currently lacking. To address this, we conducted a genome-wide identification of the *SiKCSs* in foxtail millet. We analyzed the physicochemical properties, chromosomal distribution, gene structures, and motif compositions of the family members, alongside an analysis of their evolutionary relationships and duplication events. Transcriptomic data showed that most *KCS* genes are preferentially expressed in foxtail millet seeds. These findings provide a foundational framework for research into the role of *KCSs* in grain fatty acid biosynthesis.

2 Materials and Methods

2.1 Identification of *KCS* Genes

The genomic sequences of *Setaria italica* v2.2 were retrieved from Phytozome (https://phytozome-next.jgi.doe.gov/info/Sitalica_v2_2). Known *KCS* sequences from *Arabidopsis* and rice served as queries for BLAST searches conducted via TBtools [24] and NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). In order to further verify the candidate *SiKCS* genes, the Pfam server (<http://pfam.xfam.org/>) was utilized to verify the presence of both ACP_syn_III_C and FAE1_CUT1_RppA domains. Basic physicochemical characteristics of the *SiKCS* proteins were determined using the ProtParam tool (<https://web.expasy.org/protparam/>) [25]. Furthermore, subcellular localization was predicted using WoLF PSORT (<https://wolfsort.hgc.jp/>).

2.2 Phylogenetic Analysis

MEGA was utilized to analyze the amino acid sequences across *Arabidopsis*, maize, rice, *Setaria viridis*, and foxtail millet. A phylogenetic tree was constructed via the neighbor-joining method, employing 1000 bootstrap replicates. The resulting tree data were subsequently refined and visualized using the iTOL platform.

2.3 Gene Structure, Chromosomal Location, and Motif Analysis

Chromosomal mapping data for the *KCS* genes were retrieved from the foxtail millet genome. Conserved motifs within the *SiKCS* proteins were predicted using the MEME suite (<https://meme-suite.org/>); parameters were maintained at default settings, except for the motif count, which was specified as 10, and the optimal width, which was set between 10 and 150. TBtools was employed to display the gene structures, chromosomal locations and conserved motif patterns.

2.4 Promoter Cis-Elements and Syntenic Analysis

The 2000 bp sequences upstream of each transcription start site of the *SiKCS* gene were extracted for the promoter analysis. PlantCARE was utilized to identify *cis*-acting regulatory elements within these sequences. The resulting prediction data were screened, classified, and visualized using R and Adobe Illustrator.

2.5 Synteny Analysis

TBtools was utilized for the syntenic analysis, with a focus on segmental duplications, tandem repeats, and genome-wide duplication events. BLAST searches were conducted within foxtail millet and between selected species; the resulting intraspecific and interspecific duplicated gene pairs were then used to construct covariance circles. Furthermore, the *Ka/Ks* ratios for the identified duplicated genes in foxtail millet were calculated to evaluate selection pressure.

2.6 Analysis of SiKCS Gene Expression in Different Tissues

Transcriptome data for 45 samples—comprising grains, stems, and flag leaves collected at 7, 14, 21, 28, and 35 days after anthesis—were retrieved from previous research [26]. To visualize the expression profiles of the *SiKCS* genes, a heatmap was generated using the OmicShare analytical tool (<https://www.omicshare.com/tools/home/report/reportheatmap.html>).

2.7 Subcellular Localization of SiKCS

The corresponding sequence of SiKCS28 protein was inserted into the pBI221 vector to create a fusion construct with green fluorescent protein (GFP). The resulting recombinant vectors were subsequently transformed into *Agrobacterium tumefaciens* strain GV3101. These constructs were transiently co-transformed into tobacco leaves along with the endoplasmic reticulum marker CD3-959 [27]. A Leica DMi8 confocal scanning microscope (Leica, Wetzlar, Germany) was employed to observe the infiltrated leaves and examine the transient expression patterns. All primer sequences utilized for these procedures are provided in Table S1.

3 Results

3.1 Identification and Chromosomal Distribution of the SiKCS Genes

Thirty *KCS* genes in foxtail millet were designated *SiKCS1* through *SiKCS30* according to their chromosomal locations. Except for chromosome 6, *SiKCS* genes were distributed across the remaining eight chromosomes; chromosome 9 contained nine *SiKCS* genes, whereas chromosome 8 contained only one (Fig. 1). The physicochemical properties of these genes are provided in Table S2. The average open reading frame length was 1462 bp (range: 1104–1635 bp), the average amino acid length was 486 (range: 367–544), the average molecular mass was 53,541.39 Da (range: 39,397.94–60,326.88 Da), and the average theoretical isoelectric point (pI) was 8.94 (range: 6.09–10.20). The instability index for 14 proteins was <40, which indicates that these proteins are relatively stable. Subcellular localization data indicated that 17 proteins may reside in chloroplasts, eight in the plasma membrane, three in the endoplasmic reticulum, and two in the mitochondria and cytoplasm, respectively.

3.2 Phylogenetic, Gene Structure, and Motif Composition Analysis of SiKCS Genes

The *KCS* proteins were sorted into eight different subgroups. (Fig. 2). *SiKCS* members were distributed across seven of these subclasses, with subclass η containing the highest number of proteins (10), while subclasses α and γ each contained only one protein. To determine if the structural characteristics of *SiKCS* genes vary across subclasses (Fig. 3A), the number of introns and exons for each gene was quantified (Fig. 3C). The analysis revealed that 20 *SiKCS* genes lack introns, seven contain a single intron, one contains two introns, and two contain three introns. Furthermore, motif type analysis showed that *SiKCS* proteins

possess between five and nine conserved motifs, with motif 2 and motif 8 present in all identified SiKCS proteins (Fig. 3B).

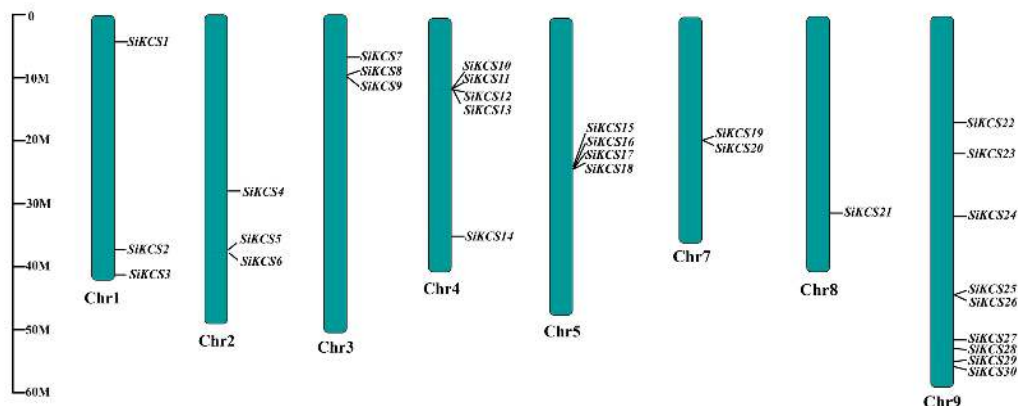


Figure 1: The chromosomal locations of *SiKCS* genes in foxtail millet.

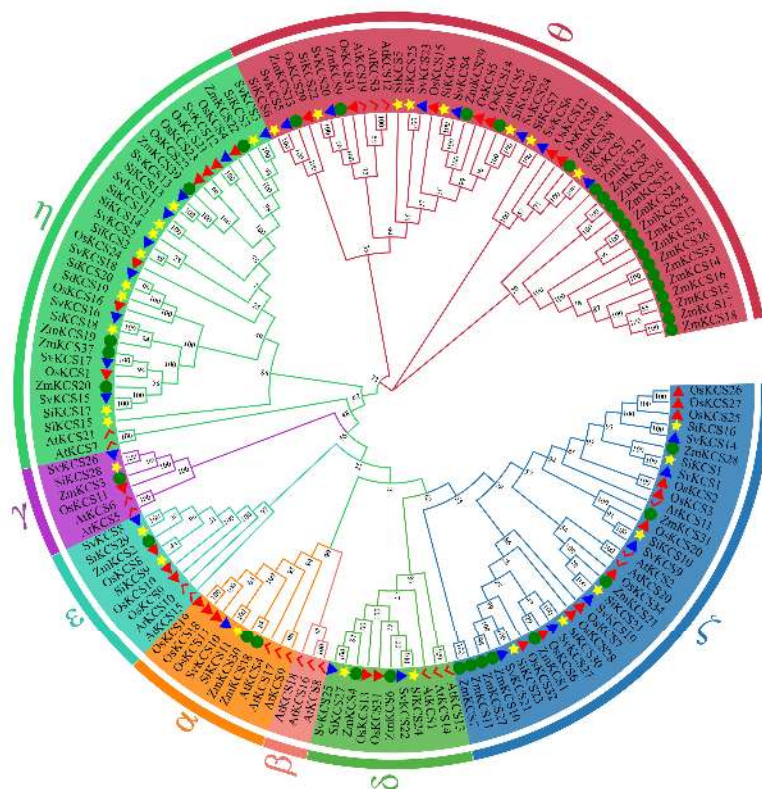


Figure 2: Phylogenetic tree of KCS proteins in foxtail millet, maize, rice, *Setaria viridis*, and *Arabidopsis*.

3.3 Analysis of *Cis*-Acting Elements in *SiKCS* Genes

The *cis*-acting elements identified within the *SiKCS* gene promoters were categorized into four functional groups: plant growth and development, phytohormone responsiveness, stress responsiveness, and light responsiveness (Fig. 4A). Elements involved in light response and phytohormone responsive represented the largest proportion, constituting 80% of the total identified sequences. Specifically, elements involved in light responsiveness (366), MeJA responsiveness (150), and abscisic acid responsiveness (145)

were the most abundant (Fig. 4B). The specific distribution of *cis*-acting elements for each *SiKCS* gene is detailed in Fig. 4C.

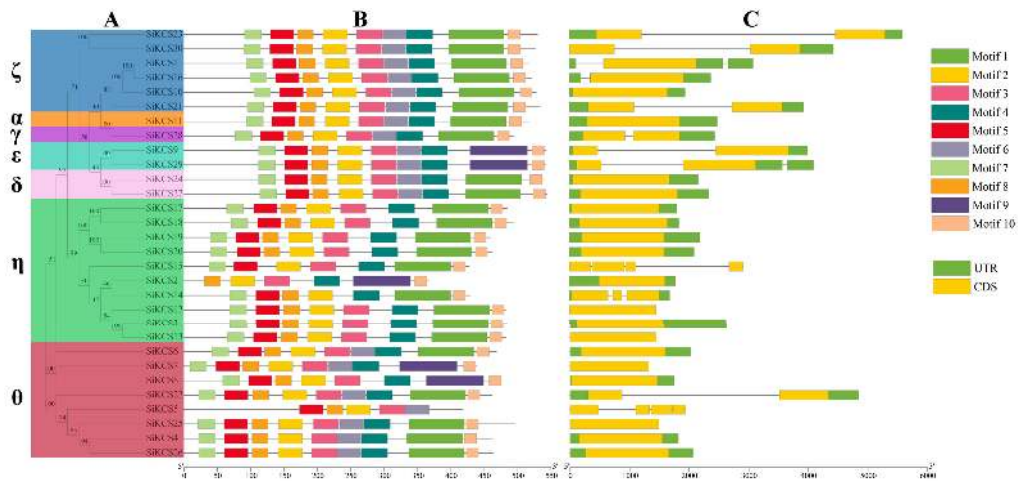


Figure 3: Phylogenetic analysis, conserved motifs, and gene structure of *SiKCS* genes. (A) The phylogenetic tree of the 30 KCS proteins identified in foxtail millet. (B) Boxes were used to depict conserved motifs, with each color indicating a different motif. (C) The structure of *SiKCS* genes is represented with yellow for exons, green for the untranslated regions, and gray for introns.

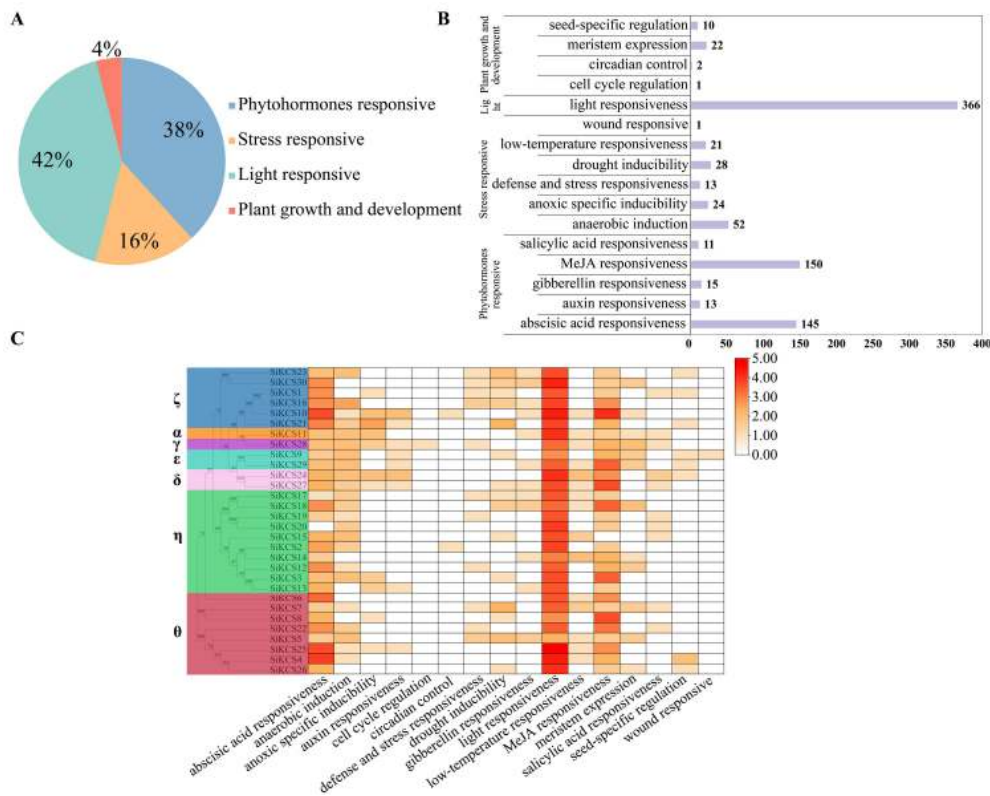


Figure 4: *Cis*-regulatory element analysis in *SiKCS* genes. (A) The percentage (%) ratio of the numerous *cis*-elements from each category. (B) The number of *cis*-acting elements per category of the *KCS* gene family. (C) Statistics for *cis*-acting elements for each *SiKCS* gene.

3.4 Synteny Analysis of *SiKCSs*

In foxtail millet, six pairs of *SiKCS* genes were identified through synteny analysis. The seven studied groups contained a total of 168 collinear gene pairs: Si–Os and Si–Sv exhibited the largest number of gene pairs (30 pairs each), followed by Sv–Os (29), Os–Zm (27), Si–Zm (26), Sv–Zm (25), and At–Os (1) (Fig. 5). The results point to a possible association between the expansion of the *KCS* members and segmental duplication events. *Ka/Ks* ratio analysis of the six duplicated *SiKCS* gene pairs revealed that four pairs possess a ratio below 0.5, while two pairs fall between 0.5 and 1.0. These data indicated that *SiKCS* genes have experienced strong purifying selection pressure during their evolution (Table S3).

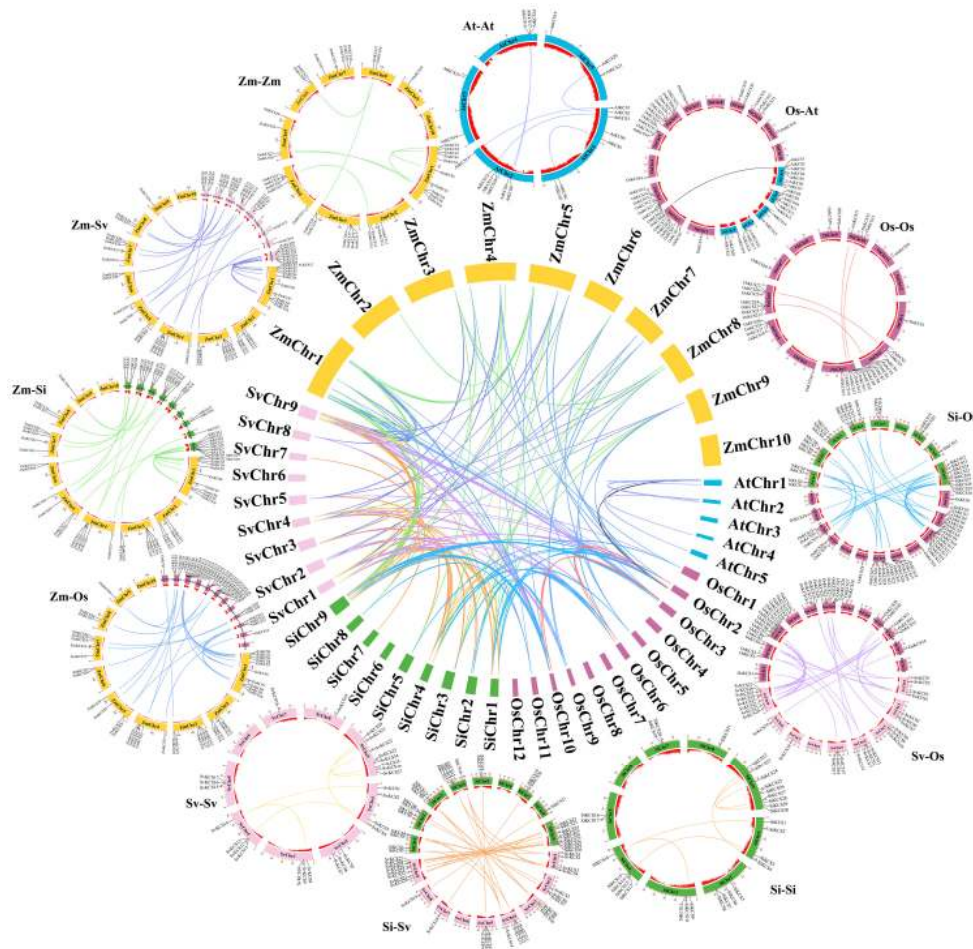


Figure 5: Collinear analysis of *KCS* genes in different crops. Chromosomes from various species are highlighted in distinct colors, with the colored lines indicating the comparative relationships both within and across species.

3.5 *SiKCS* Gene Expression Patterns in Foxtail Millet during Growth and Development

To gain insight into the possible role of *KCS* in the life cycle, we analyzed the expression of *SiKCSs* in different tissues of foxtail millet at various developmental stages (Fig. 6). Some genes are preferentially expressed in leaf and stem. But most members showed highest expression levels in seed, suggesting that *SiKCSs* may have specific functions during the process of seed formation.

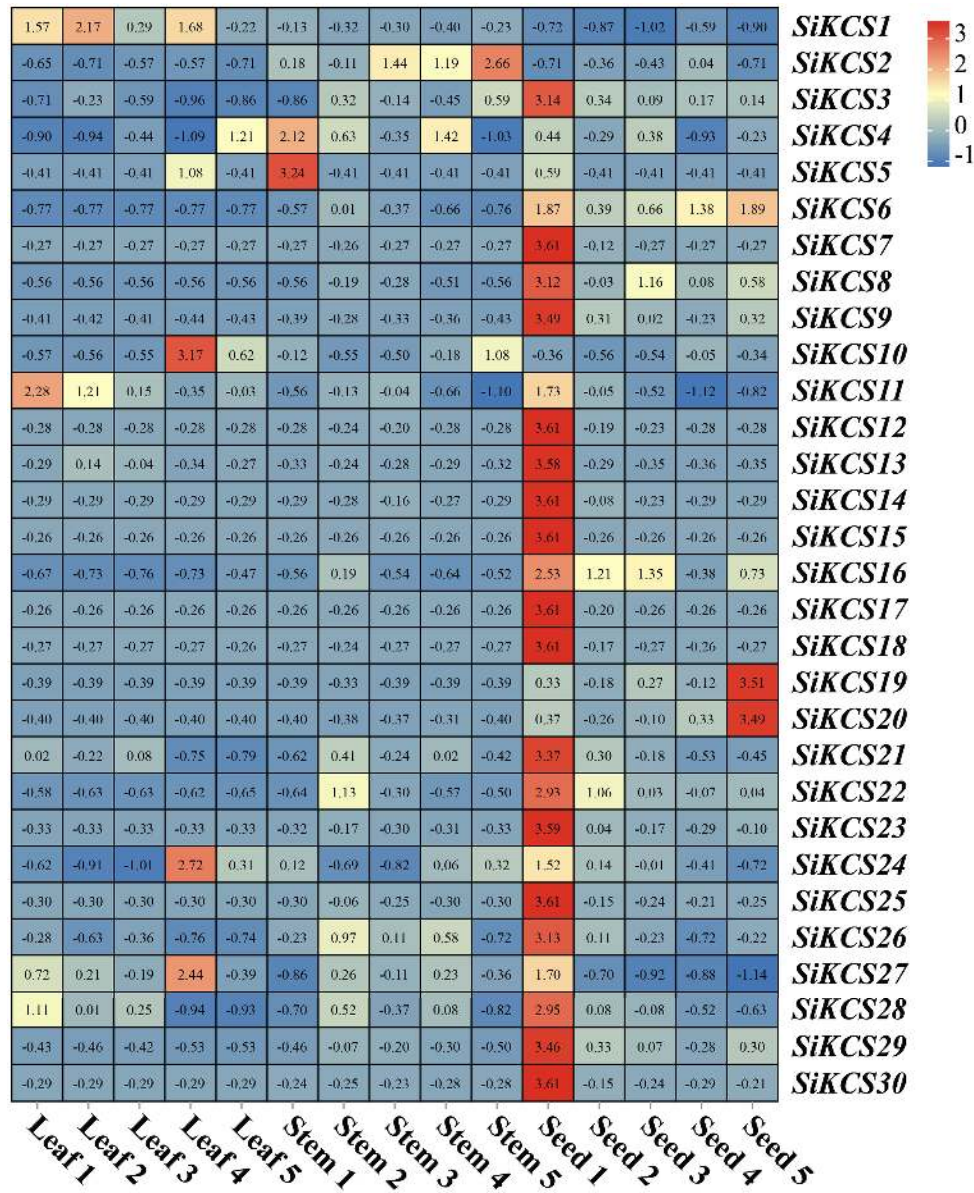


Figure 6: Expression heatmap of *SiKCS* genes across various tissues in foxtail millet at different periods. Leaf 1–5, Stem 1–5 and Seed 1–5 represent the leaf, stem and seed sampled at 7, 14, 21, 28, and 35 days after anthesis respectively.

3.6 *SiKCS* Proteins Subcellular Localization Analysis

Because the elongation of VLCFAs in eukaryotes occurs primarily in the endoplasmic reticulum via FAE complexes, *SiKCS28* was selected for further subcellular verification given its predicted localization in endoplasmic reticulum and showed high expression level in seeds. Following transient expression in tobacco cells, green fluorescence was observed within the endoplasmic reticulum using confocal laser scanning microscopy (Fig. 7). These results indicated that *SiKCS28* is localized to the endoplasmic reticulum, consistent with a functional role in FAE.

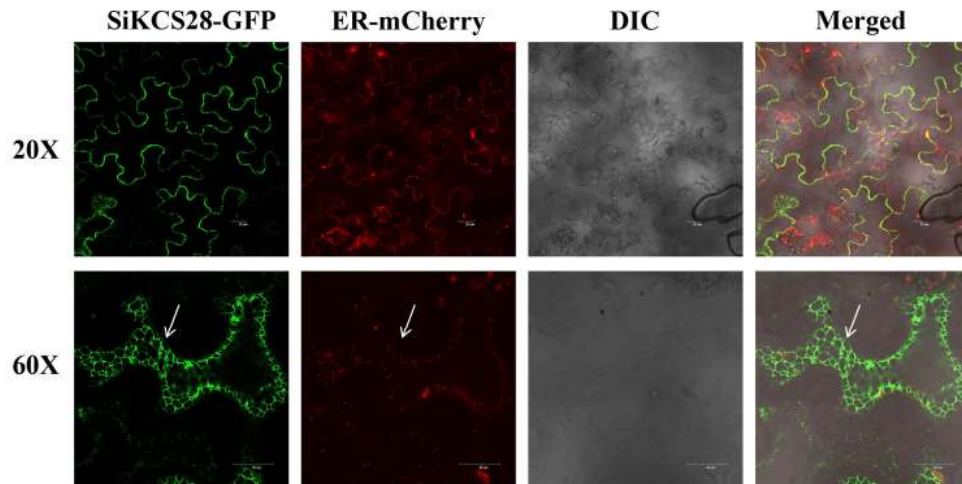


Figure 7: Analysis of the subcellular localization of GFP-fused SiKCS28 protein in tobacco cells.

4 Discussion

Seed oil is an important resource for food, industrial applications, and biodiesel production. The specific fatty acid composition of these seeds influences cell membrane fluidity and various metabolic processes [28]. VLCFAs are essential for plant growth and are indispensable for the biosynthesis of seed storage triacylglycerols, epicuticular waxes, and sphingolipids [29]. *KCS* genes play an important regulatory function in the VLCFA synthetic pathway, thereby directly affecting fatty acid profiles. Accordingly, an increasing number of *KCS* members have been characterized across diverse plant species [30–33]. The identification and functional analysis of *KCS* genes could help us gain a new sight of the regulatory mechanisms governing fatty acid accumulation and offer potential targets for the improvement of oil composition in foxtail millet.

Thirty *SiKCS* genes were identified and characterized, with distributions spanning eight of the nine chromosomes. Consistent with findings in other plant species, the *KCS* proteins of foxtail millet were classified into four subfamilies and eight subgroups [34]. Similar to the patterns observed in maize, *Setaria viridis*, and rice, the 30 *SiKCS* proteins were distributed across seven subgroups, the only exception being subgroup β , which contained only *Arabidopsis* *KCS* proteins. This indicates that *KCS* genes share a distinct and close evolutionary relationship within the monocotyledons.

Distinct conserved protein motifs can influence gene function [35]. Structural analysis of the *SiKCS* proteins revealed that motifs 2 and 8 are present across all 30 members. Subgroups ζ , α , γ , δ , and specific members of subgroup θ exhibited similar motif compositions and distributions, while subgroup ϵ shared a common motif arrangement with other members of subgroup θ . However, the motif composition of subgroup η was distinct, indicating that the *KCS* proteins within this subgroup may have undergone functional specialization. Gene structure variations are frequently associated with their expression profiles and functional evolution [36,37]. In foxtail millet, the majority of *KCS* genes contain either no introns or a single intron, a structural feature consistent with those observed in sorghum, apple, grapes, and *Arabidopsis thaliana* [31,34,38]. Furthermore, segmental and tandem duplications serve as primary drivers for gene family expansion and the acquisition of novel functions. The identification of six gene pairs on chromosomes 1, 3, 4, and 9 indicates that the expansion of the *SiKCS* gene family is likely attributable to gene duplication events, particularly segmental duplications.

Expression preference of *KCS* genes often vary among plant species. In passion fruit, the expression of most *KCS* members is highest in the fruits [30], whereas in apples, the majority of *KCS* genes exhibit peak expression in the pericarp [38]. In Brassica species, while certain *KCS* genes show increased expression in vegetative tissues, a larger number of members exhibit elevated expression levels during the onset of seed development [39]. Our findings indicated that the expression levels of most *SiKCS* genes (22/30) were highest in the seeds, particularly during the early stages of seed development. Additionally, five *SiKCS* genes showed peak expression in the leaves, and three genes exhibited their highest expression levels in the stems. Further analysis revealed that *SiKCS* genes within the ϵ and γ subgroups showed highest expression levels in seeds. Additionally, the majority of members in the η (9/10), θ (6/8), and ζ (4/6) subgroups also exhibited peak expression in the seeds (Table S4). These results indicate that *SiKCS* genes may play essential roles in seed development in foxtail millet, particularly during the early stages.

5 Conclusion

Herein, 30 *SiKCS* genes were identified within the foxtail millet, and their basic characteristics, evolutionary relationships, and expression patterns were analyzed. The *SiKCS* members can be categorized into eight subgroups, with segmental duplication identified as the primary driving force for family expansion. Expression profiling revealed that 22 genes are highly expressed in seeds, particularly during early developmental stages; such patterns suggest that most *SiKCS*s play important roles in fatty acid synthesis and grain formation. Further research will be necessary to clarify the specific biological roles of individual *SiKCS* family members.

Acknowledgement: Not applicable.

Funding Statement: This research was funded by the Key Research and Development Project of Henan Province [grant number 241111112100]; Key Research Projects of Henan Province's Higher Education Institutions [grant number 25A210025]; Enterprise Entrusted Project of Anyang Institute of Technology [grant number KYHT2023047].

Author Contributions: The authors confirm contribution to the study as follows: conceptualization: Tao Wang, Renhai Peng; methodology, investigation and data curation: Tao Wang, Hui Song; visualization: Tao Wang, Yangyang Wei, Pengtao Li; software: Yangyang Wei, Pengtao Li, Yuling Liu; writing—original draft preparation, writing—review and editing: Tao Wang, Hui Song, Renhai Peng; funding acquisition: Tao Wang. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are openly available in National Center for Biotechnology Information (NCBI) at <https://www.ncbi.nlm.nih.gov/sra/PRJNA982974>, BioProject accessions: PRJNA982974.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The supplementary material is available online at <https://www.techscience.com/doi/10.32604/phyton.2026.078858/s1>.

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