Systematic Analysis of HD-ZIP Transcription Factors in Sesame Genome and Gene Expression Profiling of SiHD-ZIP Class I Entailing Drought Stress Responses at Early Seedling Stage

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Systematic analysis of HD-ZIP transcription factors in sesame genome and gene expression profiling of SiHD-ZIP Class I entailing drought stress responses at early seedling stage

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Key message

- Drought is a key abiotic stress affecting sesame growth and productivity
- HD-ZIP transcription factors, one of the most widely studied proteins in plants drought responses, particularly HD-ZIP Class I genes
- The differential gene expression of SiHD-ZIP Class I genes in drought leads to various changes at early seedling stage in sesame
- The SiHD-ZIP Class I genes can be used to elucidate drought tolerance mechanisms in sesame

Abstract

Sesame is a very ancient oilseed crop. Sesame sensitivity to drought stress at early seedling stage is one of the limiting factors affecting its growth and yield in the world. HD-ZIP transcription factors family is one of the most important families involved in drought stress responses in plants. In this study, total sixty one sesame HD-ZIP (SiHZ) proteins were identified in sesame, based on protein sequence homology with Arabidopsis and protein domain(s) architectures were predicted by Hidden Markov model (HMM). HD-ZIP proteins were then classified into four classes (HD-ZIP Class I-IV) according to the phylogenetic, conserved domain(s) motifs and gene structure analyses in sesame. Based

on comparative phylogenetic analysis of sesame with Arabidopsis and maize HD-ZIP protein sequences, HD-ZIP Class I was subdivided into four subgroups α (SiHZ25, SiHZ43, SiHZ9 and SiHZ16), β 1 (SiHZ10, SiHZ30, SiHZ32 and SiHZ26), β 2 (SiHZ42 and SiHZ45) and γ (SiHZ17, SiHZ7 and SiHZ35). Twenty-one days old Sesame seedling were exposed to severe drought stress by withholding water for 7 days. Gene expression of 13 members of HD-ZIP Class I was performed in well-watered (control) and water stressed (treatment) seedlings. The results of gene expression analysis showed that, *SiHZ7* (6.8 fold) and *SiHZ35* (2.6 fold) from γ subgroup showed significantly high gene expression levels under drought stress in sesame seedlings. Thus, this study provides useful molecular information pinpointing the role SiHD-ZIP Class I in drought stress responses at early seedling stage and to develop sesame novel varieties with improved drought tolerance in sesame.

Key words: HD-ZIP. Transcription factors. Sesamum indicum. Queen of seeds. Drought tolerance.

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Conflicts of interest/Competing interests

The authors declare no conflicts of interest.

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Availability of data and transparency

NA

Code availability

Not applicable

Authors' contributions

Maryam Mehmood. Performed all the experiments and wrote the manuscript, **Muhammad Jadoon Khan:** Helped in gene expression analysis. **Muhammad Jawad Khan:** Helped in Real time related work. **Nadeem Akhtar:**

Helped in Bioinformatics analysis. **Fizza Mughal**: Computational assistance in HD-ZIP transcription factors identification. **Syed Tahir Abbas Shah**: Helped in statistical analysis. **Zeeshan Haider**: Technical help and provided lab facility. **Sumaira Farrakh**: supervise research, helped in finalizing the manuscript. **Irfan Sadiq**: conceived the idea, plan the research and finalize the manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Introduction

Sesame (*Sesamum indicum* L., 2n= 26) is a very ancient oil seed crop [1]. It is lauded as the “Queen of Seeds” as its seeds are a rich source of high quality and highly stable edible oil (50-60%) [2]. It is essential to human life in several ways serving as a good source of proteins (25%), carbohydrates (13.5%), polyunsaturated fatty acids, lignans (sesaminol sesamin, sesamol and sesamolinal), vitamins (B & E) and several mineral ions (phosphorous, iron, magnesium calcium, manganese, copper, and zinc) [3, 4]. Due to the pleasing aroma and taste of its seeds and oil, it is widely used for multiple purposes like cooking, confectionary, baking, livestock, medicines, and cosmetics [5]. Sesame is cultivated in arid and semi-arid lands, but its growth and yield are substantially reduced as compared to other economically important oil crops (e.g. rapeseed, peanut and soya bean) due to high temperatures and episodic/terminal drought conditions [6, 7]. Although, sesame is a well-known “survivor” or “drought tolerant” oil crop yet it is highly sensitive to drought, particularly at germination and flowering (anthesis) stage, which ultimately affect its growth and yield [3].

The identification and development of drought tolerant varieties is a crucial to sustain production of sesame crop. However, for the development of drought tolerant varieties, it is very important to understand drought tolerance mechanisms [7]. So far, only a handful molecular studies can be found on sesame drought responses, particularly at

early growth stage. Transcription factors (TFs) are one of the most important gene families participating in transcriptional regulation of drought stress responses in sesame [1].

Homeodomain-leucine zipper (HD-ZIP) is a well-characterized TF family specific to plant species. It is related to growth, development, and stress responses in plants [8]. It belongs to a very large gene family, well-known as homeobox (HB) [9]. It is characterized by a highly conserved region (~60 amino acid sequence) known as homeodomain (HD) for specific DNA binding and less conserved motif, called leucine zipper (LZ) for protein-protein interactions through homo- and/or hetero-dimerization [10]. HD-ZIP TF family is mainly divided into four classes (HD-ZIP Class I-IV) in plants [11–13].

HD-ZIP Class I which is characterized by highly conserved HD-domain and less conserved LZ motif, serves as a binding-site for dyad-symmetric sequence CAAT(A/T)ATTG [14, 15]. This class has crucial roles in plant growth, development and in abiotic stress responses. Moreover, it is also reported to be linked with cell membrane stability and abscisic acid (ABA) signaling leading to reduced plant growth specifically under drought stress [10, 13]. HD-ZIP Class II possesses CPSCE (Cys, Pro, Ser, Cys, Glu amino acids) motif and N-terminal consensus sequence, in addition to well-conserved HD-domain and LZ motif [16, 17]. Like Class I, Class II members also bind to CAAT-N-ATTG sequence but differ in the recognition of nucleotide located in the center of the target site [16]. This class is particularly involved in plant growth and development, shade avoidance, auxin response and drought stress [11]. In contrast, HD-ZIP Class III is distinguished from other HD-ZIP classes by having START domain (steroidogenic acute regulatory protein-related lipid transfer), an adjacent conserved region SAD (START-adjacent domain) and MEKHLA at C-terminal along with HD-domain and LZ motif [18, 19]. Based on in-vitro studies, HD-ZIP Class III recognizes GTAAT(G/C)ATTAC sequence as binding-site [14]. It is mainly involved in plant growth/development and oxygen redox/light signaling processes [18]. In contrast to Class I and II, role of Class III is least understood in abiotic stress like drought, salt stress and ABA treatment [11]. In HD-ZIP Class IV, HD-domain and LZ motif (loop in the middle) are also present as in Class I-III [10]. HD-ZIP Class IV also possesses START domain (lipid binding domain) like Class III which binds to AAATG(C/T)A but lacks MEKHLA domain [14]. Like Class III, Class IV has no role in abiotic stresses rather it bears functional importance in cell differentiation, flowering and cuticle development [11].

In plants, HD-ZIP Class I of HD-ZIP TF family involved in abiotic stresses, including drought. HD-ZIP Class I is further categorized into six monophyletic subgroups or clades (α , β , γ , δ , ϵ , and ϕ) in Arabidopsis [14]. HD-ZIP Class

I TFs (like *Zmhdz10* in maize) were induced by ABA treatment in drought stress and regarded as the positive transcriptional regulators to confer drought tolerance in plants [20–22]. As evident from previous studies, HD-ZIP Class I genes from γ , β and α clades showed notable response to drought stress in different plant species such as *Arabidopsis* [23] and maize [24]. In fact, depending upon plant developmental stage and environmental conditions, different gene members of this class exhibit different gene expression levels during drought stress [10]. The expression of genes from α -clade - *AtHB7*, and *AtHB12* (*Arabidopsis*), *OsHOX6*, *OsHOX22*, and *OsHOX24* (rice), *HaHB4* (sunflower), *MtHB1* (*Medicago*) and *NaHD20* (tobacco) - is typically high in drought stress demonstrating reduction in plant growth [25]. Similarly, upregulation of *ATHB5*, -6, -7 and -12 (β -clade) in *Arabidopsis* [14] and *Zmhdz7*, *Zmhdz1*, *Zmhdz3* and *Zmhdz2* (β -clade) in maize is also evident from previous studies under drought stress and ABA treatment [26]. In contrast to these, downregulation of *Zmhdz15* (β -clade) under drought stress and in ABA treatment was reported in maize [26]. Like HD-ZIP I β -clade, gene expression of HD-ZIP α -clade can also be increased (*AtHB13* and *HaHB1*) or decreased (*Zmhdz11*, *Zmhdz5* and *Zmhdz13*) upon drought stress [10].

The genes of HD-ZIP Class I (wheat *TaHDZip1-3*, -4 and -5) were characterized to specifically bind to stress-related cis-elements in the promoter sequences of drought-responsive genes to regulate drought responses [22]. In addition to this, these genes might act as upstream regulator of other TFs conferring drought tolerance in plants [10]. This type of association was previously reported between *AtHB13* (HD-ZIP Class I) and *JUB1* (a member of NAC TF family), establishing a joint drought stress control module as an upstream regulator of *JUB1* and later as a positive regulator of drought tolerance in *Arabidopsis* [10]. Moreover, altered gene expression of stress/ABA-responsive genes such as *D1-Pyrroline-5-carboxylate synthetase 1 (P5CS1)*, *Responsive to dehydration 22 (RD22)*, *Responsive to dehydration 29B (RD29B)* and *ABA insensitive1 (ABI1)* is another attribute of HD-ZIP Class I mediated drought stress tolerance, as reported in *Zmhdz10* over-expressors transgenic rice plants [21].

So far, genome-wide analyses have been performed on a number of stress related TF families such as ERF, WRKY, MYB, NAC and bZIP in sesame [27]. Moreover, genome-wide classification of HD-ZIP gene family and their expression analysis under drought and salinity stress in different tissues (root, stem, flower, leaf, capsule and seed) of sesame has already been reported [27]. In the current study, the classification of HD-ZIP proteins in sesame has not only extended from its four well-conserved classes to further subgrouping of Class I into α , β , γ and δ but also possible role of α , β , γ and δ was explored in severe drought

stress response of sesame at early stage. To our knowledge, the role of HD-ZIP Class I in drought stress responses entailing early growth stages in sesame has not been studied so far.

Materials and Methods

In silico identification of SiHD-ZIP proteins

HD-ZIP protein sequences were identified from sesame whole proteome (*S_indicum_v1.0*), downloaded from National Center for Biotechnology Information (NCBI: <https://www.ncbi.nlm.nih.gov/genome/>). This identification was based on typical homology search and prediction of protein domain architecture using The HMMER approach. First of all, homology search was performed between sesame whole proteome sequences with that of protein sequences of *A. thaliana* transcription factors families, retrieved from The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org/>) to create a database for homology search on BLAST standalone version (2.6.0+). The candidate TFs orthologs in sesame were predicted based on reciprocal BLASTP hits defined as: $\geq 80\%$ query coverage, $e\text{-value} < 10^{-6}$ and $\geq 50\%$ sequence identity. From the set of identified TFs orthologs in sesame, redundant protein data (duplicate hits) was eliminated using python scripting. Thus, obtained non-redundant protein sequences with query coverage $\geq 80\%$ were selected as candidate transcription-associated proteins (TAPs) in sesame. Candidate TAPs were verified based on their domain(s) architecture using Superfamily (1.75) based on HMM library comprising Hidden Markov's Models [12] and python scripting. After domain(s) verification, the proteins were classified into plant-specific TF and TR families in sesame, according to a consensus rule based on previously established methods for domain(s) assignment in protein sequences, as reported by [28]. Total number of well-characterized HD-ZIP proteins as well as their respective gene loci, linkage groups (LG1-LG16), chromosomal locations and exon counts from NCBI were determined in sesame. Subsequently, based on this information, a numerical name was designated to each of these predicted SiHD-ZIP protein sequences depending upon their distribution order (LG1-LG16) in sesame.

Phylogenetic analysis and classification of SiHD-ZIP proteins

After removing unassigned and outlier protein sequences from the analysis, the predicted SiHD-ZIP proteins were selected for their classification into four well-conserved classes of HD-ZIP TF family known in plants. Multiple sequence alignments were built by ClustalW (<http://www.clustal.org/>). Then, a phylogenetic tree was constructed by choosing maximum likelihood (ML) method and 1000 bootstrap replications in order to divide them into four classes (SiHZ I-IV) using MEGA (version X) [29]. For the sub-classification of SiHD-ZIP Class I into subgroups and clades, second ML tree was built from the multiple sequence alignment of protein sequences of SiHD-ZIP Class I and already-

reported members of SiHD-ZIP Class I in maize and Arabidopsis [26]. After construction, the phylogenetic trees were analyzed using Dendroscope version 3.7.2 [30].

Identification of conserved domain(s), isoelectric point (pI)/molecular weight (kDa) and isoelectric point (pI) in SiHD-ZIP Class I-IV

To validate the classification of SiHD-ZIP Class I-IV protein sequences, the conserved motifs of these proteins were determined by subjecting protein sequences to online MEME (Multiple Expectation Maximization for Motif Elicitation) version 5.1.1 with parameters set as: optimum motif width (≥ 6 and ≤ 200); maximum number of motifs (20) [31]. The conserved motifs were then further verified by two conserved motifs prediction tools, Expasy PROSITE [32, 33] and Interpro 82.0 [34]. Moreover, domain architectures of HD-ZIP Class I-IV proteins were also predicted by SMART (Simple Modular Architectural Research Tool), to confirm the presence of class-specific domains in SiHD-ZIP protein sequences [35]. The isoelectric point (pI)/ molecular weight (kDa) of each of these proteins were calculated by Expasy online tool https://web.expasy.org/compute_pi/.

Gene structure analysis of SiHD-ZIP proteins

The gene structure analysis of SiHD-ZIP Class I-IV was performed by Gene Structure Display Server (GSDS) version 2.0 [36]. To determine their gene structures, the coding and genomic sequences of these proteins were obtained from NCBI database.

Plant material and drought stress treatment

Sesame seeds of TS-5 variety were collected from National Agricultural Research Centre (NARC), Islamabad, Pakistan. After overnight soaking (~24 hours) in fresh water, seeds were dried and sown in plastic pots filled with a mixture of soil, sand and peat in 2:1:1 ratio and kept at $25 \pm 3^\circ\text{C}$ with 16-h day, 8-h night photoperiod. After 20 days of seedling growth, drought stress was imposed by complete water withholding in drought treated seedlings. The control plants were watered regularly throughout the experiment. The leaf samples were subsequently collected from both control and drought stressed seedlings when the soil moisture reached 16%. After collection, each leaf sample was immediately treated with liquid nitrogen for subsequent RNA extraction and qPCR. The experiment was performed with three independent biological replicates.

RNA extraction and quantitative polymerase chain reaction (qPCR) analysis

For the gene expression analysis of *SiHD-ZIP* Class I genes under drought stress, total RNA was extracted from the leaf samples of control and drought stressed seedlings using Trizole method [37]. The integrity of the isolated RNA was assessed by 1% denaturing agarose gel. The concentration of isolated RNA was determined by a spectrophotometer (Analytik Jena SPECORD 200 plus). After RNA quantification, each sample was further processed with DNase 1 (Thermo Fisher Scientific) to remove DNA contamination. The DNA-free RNA sample was converted into cDNA by using a cDNA synthesis kit (Thermo Fisher Scientific). Gene-specific primers for qPCR were designed for the identified genes of HD-ZIP Class I by using the NCBI primer designing tool (<https://www.ncbi.nlm.nih.gov/tools/primer>) and are listed in Supplementary Table S3. The qPCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) at 54 °C. The actin gene was used as reference gene and three independent biological replicates for control and drought stressed seedlings were used to carry out the gene expression analysis by qPCR. Relative fold-change of target genes was determined by $2^{-\Delta\Delta C_t}$ method as described by [38].

Statistical analysis

For statistical analysis one-way ANOVA was performed followed by Tukey's test at significance level $P = 0.05$ using Origin 2019b (<https://www.originlab.com/index.aspx?go=Products/Origin/2019b&pid=3322>). The results were expressed as mean $\pm$ standard error (SE) of three replicates.

Results

In-silico identification of SiHZ proteins

In the given study, total 61 well-annotated HD-ZIP proteins were predicted in sesame genome. After prediction, names were assigned to these protein sequences (SiHZ 1-45) according to their chromosomal locations on sesame linkage groups (LGs) (Supplementary Table S1).

Phylogenetic analysis of HD-ZIP proteins and their classification in sesame

Based on the phylogenetic analysis, the identified HD-ZIP protein sequences were classified into four classes (Figure 1). The resulting phylogenetic tree (Maximum likelihood (ML) tree) reveals the distribution of selected SiHZ proteins into four classes: Class I (13), Class II (12), Class III (21) and Class IV (14) (Fig. 1).

Subsequently, an ML tree was also constructed for further division of HD-ZIP Class I into its clades or sub-groups (α , $\beta 2$, $\beta 1$, ζ , δ , ϵ , γ and ϕ) as shown in Fig. 2. In Class I, the highest number of protein members fall into α sub-group (6). Out of these six members, SiHZ10, SiHZ30, SiHZ32 and SiHZ26 were clustered in $\alpha 1$ while SiHZ42 and SiHZ45 in $\alpha 2$ (Fig. 2). Besides these, four members (SiHZ25, SiHZ43, SiHZ9 and SiHZ16) were included in β sub-group followed by three members (SiHZ17, SiHZ7 and SiHZ35) in γ sub-group (Fig. 2). None of the members of Class I were found in subgroups ζ , δ , ϵ and ϕ in sesame (Fig. 2).

Sesame HD-ZIPs conserved domains, their isoelectric points (pI)/molecular weight, MW (kDa) in Class I-IV

In sesame, for validation of HD-ZIP proteins (Class I-IV), their conserved domains were identified by MEME tool (Fig. 3). Total 20 conserved domains were identified in SiHZ proteins, ranging from 20 to 98 amino acids in length (T). Then, conserved amino acid sequences of these domains were used to confirm domain's presence and its molecular function in all four classes with the help of Expasy Prosite and InterPro (Supplementary Table S2). From Expasy Prosite, only Homeobox domain was predicted in Motif 1 (common feature of all four classes of HD-ZIP proteins) and START domain in Motif 4, which is a characteristic feature of both, Class III and IV (Supplementary Table S2). The selected protein sequences were analyzed with InterPro. Analysis by InterPro showed greater number of predicted conserved domains and their molecular function to validate HD-ZIP proteins in all four classes in sesame i.e. Motif 1, 3, 4, 5, 8, 9 and 11 (Supplementary Table S2). The presence of specific domains belonging to four classes was confirmed by SMART (Fig. 4). According to results, HOX domain was found in all Classes (I-IV), HALZ domain was exclusively identified in Class II, START domain was found both in Class III and IV but MEKLA domain was only present in Class III (Fig. 4).

Furthermore, number of amino acids (a.a), isoelectric point (pI)/molecular weight (MW) of these selected proteins were also analyzed. The result showed great variation among member proteins belonging to each class in their amino acids sequence length, molecular weight and isoelectric point (Table 1). In sesame, HD-ZIP proteins ranged from a minimum of 201 (SiHZ7 from Class I) to a maximum of 853 (SiHZ41b from Class III) amino acids in length. Among SiHZ proteins, the largest amino acids sequence length was obtained in Class III ranging from 803 a.a in SiHZ22c to 853 a.a in SiHZ41b while the shortest range was found in the members of Class I from 201 a.a (SiHZ7) to 340 a.a (SiHZ30). Likewise, the pI values were high in Class III and ranged from 5.81 (SiHZ34) to 6.28 (SiHZ13) as compared rest of the classes. In Class IV, it ranged from 5.40 (SiHZ6a, b) to 8.50 (SiHZ12b) while in Class II ranging from 4.99

(SiHZ3a, b,) to 8.74)] and 4.73 (SiHZ10) to 7.71 (SiHZ7) in Class I (Table 1). Similarly, the range of molecular weights (kDa) was determined highest in Class III members followed by Class IV, II and I (Table 1).

Sesame HD-ZIPs chromosomal locations and their gene structure analysis in Class I-IV

In sesame, an uneven distribution of SiHD-ZIP genes was found, located over 1-12 LGs (Table 1). Among these genes, total 13 genes of Class I were unevenly distributed on six different LGs (2, 4, 8, 11, 13 and 15) while 10 genes of Class II were located on LGs 1-4, 6, 11 and 15 (Table 1). Similarly, genes in Class III and IV, each comprising 8 and 9 genes, respectively, were located on LGs (1,3,4,6-9 and 12) and (1-4 and 8), respectively (Table 1). The greatest number of genes (9) in Class IV were found distributed over lesser number of LGs (1-4 and 8) as compared to Class I-III (Table 1). Moreover, LG 4 was found common to all classes having two genes from Class I and one of the gene members of Class II-IV (Table 1). Besides this, LG 13 was only common to Class I and LGs (7, 9 and 12) were only identified in Class III (Table 1).

Likewise, the gene structure analysis of SiHD-ZIP genes showed great variability in the number of introns ranging from 1-17 (Fig. 5). The results showed genes of each class were quite similar in exon-intron structure and clustered together. The number of introns in Class I and II were comparatively lesser than Class III and IV. In Class I, majority of the genes had two introns except for *SiHZ 7*, *SiHZ17* and *SiHZ35* which had only one intron in their genes and *SiHZ26* had three introns. Likewise, most of the genes had three introns in Class II but *SiHZ14* and *SiHZ44* were the genes with two introns. Out of these genes, the highest number of introns (17) was found in genes of the Class III while the number of introns in Class IV genes was comparatively greater than Class I & II but lower than Class III, ranging from 8-10 introns (Fig. 5).

Effect of drought stress on sesame seedlings

Under severe drought stress condition, sesame seedlings showed reduced or slow growth as compared to control (well-watered) seedlings in sesame (Fig. 6). In addition to slow plant growth, curling of leaves with mild yellowing were also observed in drought treated seedlings (Fig. 6).

Expression analysis of HD-ZIP Class I genes under drought stress at early seedling stage in sesame

In this study, gene expression analysis of 13 genes of SiHD-ZIP Class I was performed in seedling under severe drought stress along with well-watered control. Out of these 13 genes, 8 genes showed differential expression under

drought stress. The results demonstrate significantly high upregulation of two genes *SiHZ7* (6.8 fold) and *SiHZ35* (2.6 fold) from □□ subgroup while downregulation of two other genes (*SiHZ25* with fold change ~0.76 and *SiHZ43* having fold change ~0.59) of □□ subgroup (at $P < 0.05$) in severe drought stressed as compared to control seedlings (Fig. 7). In addition to this, the expression of rest of the selected genes i.e. *SiHZ9* and *SiHZ16* (□); *SiHZ10*, *SiHZ26*, *SiHZ30* and *SiHZ32* (□1); *SiHZ42* and *SiHZ45* (□2) and *SiHZ17* (□) showed no significant difference in the gene expression levels between drought stressed and control seedlings (Fig. 7).

Discussion

In-silico identification of SiHD-ZIP proteins

HD-ZIP is a plant-specific TF family known for its roles in plant growth, development and responses to light and stress [39]. So far, HD-ZIP is a widely characterized TF family throughout plant kingdom such as Arabidopsis (48) [14], rice (48) [40], maize (55) [26], wheat (46) [22], potato (43) [39], tomato (51) [41], medicago (52) [42], foxtail millet (47) [20], tea plant (33) [8], grape (31) [43], cassava (57) [11], soybean (88) [44], Chinese cabbage (113) [45], poplar (63) [46], pear (52) [47], peach (33) [48], plum blossom (32) [49] and eucalyptus (40) [50]. In the given study, total 61 sesame HD-ZIP proteins, encoded by comparatively smaller number of genes (44) have been identified, based on sequence homology search (*A. thaliana*) and domains assignment rules by HMM (Supplementary Table 1). In the light of previous studies, results showed that species bias does exist in the distribution of HD-ZIP genes and it varies from one plant species to another [46]. The HD-ZIP genes are most likely to evolve and expand in numbers due to segmental gene duplication events in different plant species [27]. Thus, the number of HD-ZIP genes in sesame (44) found in this study was almost similar to that of potato (43) [39]. In contrast to this, the highest number of HD-ZIP genes has been reported in Chinese cabbage (113) [45] and soybean (88) [44, 51]. Thus, these findings shed light on the evolutionary history of sesame as well. From evolutionary point of view, it is distantly related to other oil crops such as soybean, castor and rape but closely related to potato (*Solanaceae* family) and *Phrymaceae* families in the core lineage of eudicots-angiosperms [52]. Thus, the number of HD-ZIP genes in sesame sharing similarity with potato but greatly differing from soybean is providing an interesting clue about the evolutionary relationship of sesame with other oilseed crops.

Phylogenetic analysis of SiHD-ZIP proteins and their classification

In this study, a phylogenetic analysis was performed for the classification of sesame HD-ZIP proteins into four well-conserved classes known in plants (Fig. 1). The clustering of similar HD-ZIP members within the same class in sesame is clear from this phylogenetic analysis (Fig. 1). These results clearly reflect high conservation of HD-ZIP TF family in plants and agree with previously classified HD-ZIP TFs in other plant species like Arabidopsis [14], maize [26], potato [39] and eucalyptus [50].

In addition to this, sesame HD-ZIP Class I was further divided into subgroups in this study. The subgroups assignment of member proteins in Class I was done based on their clustering within already known subgroups (α , β 2, β 1, ζ , δ , ϵ , γ and ϕ) in Arabidopsis and maize (Fig. 2). According to results, clades α , β 2, β 1 and γ were common to all three plant species selected for the phylogenetic analysis. In previous studies, some of these subgroups were shown to be lineage-specific such as α clade (subgroup) is exclusively shared among eudicots (Arabidopsis, Medicago, grape, and poplar) while clade β is common to monocots (rice, millet, and stiff brome) and ϕ clade to moss [46]. It is clear from these results that these subgroups were neither found in sesame nor Arabidopsis and maize. In addition to these, sesame also lacks clade δ which was common to both Arabidopsis and maize as well as clades ϵ , and ϕ which were only common to Arabidopsis [24]. So, the subgrouping within the Class I found to be consistent with previously reported studies. In Fig. 2, the presence of α clade and absence of clade β in sesame and Arabidopsis as compared to maize (monocot) ensures their eudicot lineage. It is most likely that sesame might share more functional similarity with Arabidopsis HD-ZIP I class in transcriptional regulation of genes during growth, development and/or abiotic stress responses, including drought.

SiHD-ZIP Class I-IV conserved domains and their isoelectric points (pI)/ molecular weight (Da)

In the given study, high consensus was found in the conserved domain(s) architectures of member proteins within each class, represented by their respective member as shown in Fig. 6. According to results, the domain(s) architectures were common to all members within each class of SiHD-ZIPs and were supported by previous findings [53, 54]. Likewise, all SiHD-ZIP members within each class shared great similarity in the values of their amino acid number, pI/MW (Table 1). Overall, members of Class I and II have shorter amino acids sequence length as compared to Class III and IV (Table 1). Similarly, there was more difference in pI values within members of Class I and II as compared to Class III and IV in sesame. Thus, these results clearly reflect same motif organization within the same class but different motif organization among all four classes in SiHD-ZIPs. Hence, these results clearly demonstrate class-specific diversity in the protein sequence, structure and function of HD-ZIP TF family in sesame.

SiHD-ZIP Class I-IV chromosomal locations and their gene structure analysis

According to results, there was an uneven distribution of *SiHD-ZIP* genes located on 1-12 LGs in sesame (Table 1). These results represented the highest number of gene distribution on LG 8 [10 genes: Class I (3), Class III (1) and Class IV (5)] in sesame (Table 1). In contrary, the lowest gene distribution of *HD-ZIP* genes was found on LGs 7, 12 and 13 i.e. only one gene per LG (Table 1). Moreover, the gene structure analysis of *SiHD-ZIP* genes also revealed similar gene structure organization (gene size, number of introns and exons as well as their lengths) shared among members of each class (Fig. 5). Thus, these findings clearly suggest that *SiHD-ZIP* genes located on the same LG (chromosome) are most likely to be linked with each other. Thus, it is inferred that these linked genes might function together in transcriptional regulatory gene networks involving plant growth, development, metabolic pathways and/or or stress responses in sesame.

This is another inevitable finding of this study that the number of *SiHD-ZIP* genes (44) identified in this study was quite close to the previously reported number of *SiHD-ZIP* genes (45) in sesame (45) [27]. According to results, the number of identified SiHD-ZIP proteins (61) was much greater than the number of total genes (44) in sesame (Table 1). In this respect, HDZIP Class III is the largest class having the greatest number of SiHD-ZIP proteins (21) encoded by only 9 genes in sesame (Table 1). Similarly, Class IV has total 14 protein members, encoded by only 7 genes and Class II comprises of 12 member proteins encoded by 10 genes (Table 1). In contrast to them, the 13 member proteins are encoded by 13 different genes in Class I (Table 1). Such comparative analysis between SiHD-ZIP proteins (splice variants or protein isoforms) and their respective genes provides useful information to elaborate the functional diversity of HD-ZIP TF family and organism's complexity in sesame [55, 56]. So, in the given study, some of the HD-ZIP proteins in sesame are possible variants (protein isoforms) of the same *HD-ZIP* gene which can be formed at a time depending upon the tissue type or stage of development [57]. These results are consistent with previously reported studies e.g., the number of *HD-ZIP* genes found in potato were 15, 10, 7 and 9 in Class I-IV, respectively [39], which is quite similar to the results reported in this study. In contrary, *Arabidopsis* and maize, both of them have the least number of genes (5) in HD-ZIP Class III while more genes members belong to HD-ZIP Class II (18) and HD-ZIP Class I (17) followed by HD-ZIP Class IV (15) in *Arabidopsis* and 17, 16 and 10 in Class I, IV and II, respectively, in maize [26]. Moreover, a good comparison can be made between SiHD-ZIP Class I-IV proteins and soybean in which the largest number of HD-ZIP proteins falls in Class I (30) and II (27) while least in the Class III (12) and 19

in class IV soybean [51]. Thus, if the comparison of these classes is made based on number of genes, more structural and functional diversity can be linked in the genes of Class I. Thus, the functional diversity in sesame's HDZIP TF family can be implicated by two means, either through the expansion of the Class I by segmental gene duplication events [27] or alternative splicing of genes to produce multiple splice variants (protein isoforms) from a single or few genes of Class III, IV and II respectively [56].

Effect of drought stress on sesame seedling growth

It is evident from results that sesame seedlings did not show wilting rather resumed slow plant growth with slight yellowing and curling of leaves (Fig. 6). As evident from previous findings, this reduction in growth can be linked to some molecular alterations (e.g. over-expression of member gene(s) from HD-ZIP Class I) to confer drought tolerance in sesame at seedling stage [53].

Expression analysis of HD-ZIP Class I genes under drought stress at early seedling stage in sesame

The functional relevance of SiHD-ZIP Class I in drought and other abiotic stress responses is widely reported in several plant species like Arabidopsis [53], sunflower [58] and tobacco [59]. Recently, significantly high expression of HD-ZIP I genes (*MtHDZ17*, *MtHDZ34*, *MtHDZ35*, *MtHDZ39*, *MtHDZ47*, *MtHDZ48*, and *MtHDZ50*) along with several members of HD-ZIP Class II has been reported in response to drought and salinity stress treatments in medicago [42]. However, changes in HD-ZIP Class I gene expression are inevitable, induced by water-limiting conditions or ABA treatment. In cassava, a dramatic change in the expression of Class I genes in leaves under drought stress suggests longer periods of drought could cause dramatic effects on cassava [11]. Similarly, both, upregulation or downregulation of Class I genes at different time points (6hr, 12hr & 24hr) have also been reported in soybean [44]. According to the results of this study, members of Class I showed either upregulation, downregulation or no change in their gene expression in the drought treated seedlings compared to the control (well-watered) seedlings (Fig. 6). Similar pattern of gene expression was observed in previous studies in other plants such as tobacco, where HD-Zip class 1 genes showed similar trends in response to drought stress [59].

In the given study, *SiHZ7* and *SiHZ35*, two members of subgroup α subgroup exhibited significant upregulation under drought stress. Similarly, as previously reported in maize, the gene expression levels of subgroup γ (*Zmhdz12*, -9, -6 and -4) were strongly induced by more than 2-fold compared to the control [26]. Such high gene expression of members from α clade is typically induced by ABA treatment and water deficit conditions and have previously reported in Arabidopsis (*AtHB7*, *AtHB12*), sunflower (*HaHB4*), medicago (*MtHB1*), tobacco (*NaHD20*), maize

(*ZmHDZ4*) and rice (*OsHOX6*, *OsHOX22*, and *OsHOX24*) enhancing drought and salinity tolerance via ABA signaling in transgenic plants [27]. Albeit, *SiHZ7* and *SiHZ35*, are the two members of the same subgroup $\square$, showed high gene expression levels under drought stress in this study. They share great similarity with each other as two paralogs yet their divergent evolution and function can be speculated in order to mediate growth responses under water deficit conditions as previously reported in Arabidopsis (*AtHB7* and *AtHB12*) [60, 61]. In the light of previous findings, reduction in cell elongation and plant growth can be inferred with the upregulation of *SiHZ7* and *SiHZ5* in sesame under drought stress [53]. Most probably, this reduction in cell elongation and subsequent drought tolerance in sesame can be either due to inhibition of GA biosynthesis such as *AtHB12* in Arabidopsis or without the inhibition of GA biosynthesis like *AtHB7* in Arabidopsis [53]. In addition to this, like *AtHB13* (Arabidopsis), either both or any of these two TFs (*SiHZ7* or *SiHZ35*) may confer drought tolerance at early seedling stage by acting as upstream positive regulator(s) of other TFs such as NAC (*JUB1* or its homolog) in sesame [53]. Thus, it can be speculated that *SiHZ7* and *SiHZ35* may work in a coordinated fashion by changing their gene expression levels to fine tune processes associated with growth (early seedling stage) and water stress in sesame [62]. However, functional analysis of *SiHZ7* and *SiHZ35* is required to draw a better picture of gene or metabolic network(s) involved in sesame drought stress responses/tolerance at early seedling stage.

On the other hand, two members of subgroup $\square$ (*SiHZ25* and *SiHZ43*) showed their downregulation in drought seedlings as compared to control. These results seem quite similar with the expression of HDZ-I genes from-clade in maize [24]. According to [24], drought stress resulted in the downregulation of *Zmhdz11*, -5 and -13 of subgroup $\square\square\square$ while the expression levels of the subgroup γ genes (*Zmhdz12*, -9, -6 and -4) were strongly induced more than 2-fold as compared to the control plant [24]. In contrast to this, *AtHB13* (Arabidopsis) and *HaHB1* (sunflower) were found to constitutive overexpression of $\square\square\square$ clade members with no significant effect on the growth and yield of transgenic Arabidopsis under drought stress [63]. However, their high expression levels may induce plant stress tolerance to cold, drought and/or salinity by enduring cell membrane integrity and stability in plant cells [63]. In the given study, no significant gene expression changes were found in case of subgroup $\square$ under drought stress, particularly, *SiHZ26* and *SiHZ32* ($\square 1$) and *SiHZ42* ($\square 2$). In contrast to these results, either up- or downregulation of subgroup $\square$ gene members is evident in different plant species from previous studies. In Arabidopsis, elevated levels of *ATHB5*, *ATHB6*, *ATHB7*, or *ATHB12* were induced in response to ABA and water deficit conditions to regulate plant growth [14]. Likewise, high expression levels of *Zmhdz7*, *Zmhdz1*, *Zmhdz3* and *Zmhdz2* of subgroup $\square 1$ were

induced while the expression of *Zmhdz15* was greatly downregulated by drought stress in maize [26]. Besides this, under drought stress, the downregulation of Class I members such as *SiHZ25* and *SiHZ43* (subgroup □) can be linked to an increase in plant water content and malondialdehyde content to enhance drought tolerance in sesame [59]. Thus, it is most likely that *SiHD-ZIP* Class I genes may function in a coordinated manner, particularly, *SiHZ7* and *SiHZ35* from subgroup □□□ in the developmental reprogramming of sesame at early seedling stage in response to drought stress, as already reported in Arabidopsis [64]. Thus, these results clearly suggest both upregulation and downregulation of *SiHD-ZIP* Class I gene members under drought stress responses at seedling stage in sesame. However, it is most likely that gene members of *SiHD-ZIP* Class I belonging to subgroups □□ and □□ have more functional importance to regulate early drought stress responses in sesame.

Conclusion

This study reveals differential gene expression patterns of *SiHD-ZIP* Class I under drought stress in sesame seedlings. Sesame is known for its sensitivity to drought at early seedling stage. Therefore, this study provides meaningful information to extend our understanding on sesame drought responses at early seedling stage. In the light of results, *SiHDZIP* Class I members from subgroup □□□ (*SiHZ7* and *SiHZ35*) can be considered good candidate genes to have significant role in drought stress developmental reprogramming of early growth stages under drought stress in sesame. Thus, the given study may help to reveal the *SiHD-ZIP* Class I mediated regulatory networks of drought stress response at early seedling stage in sesame. Moreover, these findings may provide baseline molecular information to design new strategies to engineer sesame novel varieties with improved drought tolerance.

Declarations

This manuscript present original research.

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Table 1 List of HD-ZIP proteins classified in sesame: their amino acid sequence length (a.a), theoratical iso-electric point (pI)/molecular weight (MW, kDa) and their gene distribution on different linkage groups (LGs)

SiHDZI P name	Protein accession id	Amino acid sequence length (a.a)	Theoratical pI/MW(kDa)	Gene symbol id	Linkag e group (LG)	Exon coun t	Clas s
SiHZ1	XP_011075631.1	221	6.02/24.8	LOC105160061	LG1	4	II
SiHZ2	XP_011080516.1	845	5.96/92.4	LOC105163757	LG1	18	III
SiHZ3a	XP_011087051.1	364	4.99/40.1	LOC105168612	LG1	5	II
SiHZ3b	XP_011087055.1	363	4.99/40	LOC105168612	LG1	5	II
SiHZ4	XP_011089804.1	304	8.46/34	LOC105170646	LG1	4	II
SiHZ5	XP_011091721.2	837	6.47/91.7	LOC105172078	LG1	9	IV
SiHZ6a	XP_011070327.1	817	5.40/90.6	LOC105156012	LG2	13	IV
SiHZ6b	XP_011070328.1	817	5.40/90.6	LOC105156012	LG2	13	IV
SiHZ6c	XP_011070329.1	816	5.40/90.5	LOC105156012	LG2	13	IV
SiHZ7	XP_011070385.1	201	7.71/23	LOC105156051	LG2	2	I
SiHZ8	XP_011070725.1	212	8.74/24	LOC105156327	LG2	4	II
SiHZ9	XP_011071017.1	292	5.96/33.2	LOC105156551	LG2	3	I
SiHZ10	XP_011071034.1	316	4.73/36	LOC105156564	LG2	3	I
SiHZ11	XP_011072039.1	717	6.30/79.1	LOC105157339	LG3	10	IV
SiHZ12a	XP_011072373.1	757	6.50/84.2	LOC105157647	LG3	11	IV
SiHZ12b	XP_020548412.1	659	8.50/73.9	LOC105157647	LG3	11	IV
SiHZ13	XP_011072422.1	840	6.28/92.3	LOC105157678	LG3	18	III
SiHZ14a	XP_011073660.1	269	8.71/29.9	LOC105158569	LG3	4	II
SiHZ14b	XP_011073661.1	242	8.63/26.8	LOC105158569	LG3	4	II
SiHZ15	XP_011075258.1	325	7.58/36.2	LOC105159770	LG4	4	II
SiHZ16	XP_011075470.1	307	6.05/34.8	LOC105159939	LG4	3	I
SiHZ17	XP_020549042.1	220	5.21/25.9	LOC105160292	LG4	2	I
SiHZ18	XP_011076208.1	811	6.48/88.5	LOC105160506	LG4	9	IV

SiHZ19a	XP_011076980. 1	836	5.93/91.8	LOC10516109 9	LG4	18	III
SiHZ19b	XP_011076981. 1	835	5.86/91.7	LOC10516109 9	LG4	18	III
SiHZ19c	XP_011076983. 1	833	5.93/91.5	LOC10516109 9	LG4	18	III
SiHZ20	XP_011081291. 1	307	8.51/34.1	LOC10516435 5	LG6	4	II
SiHZ21	XP_011081776. 1	841	6.00/92.8	LOC10516473 3	LG6	19	III
SiHZ22a	XP_011084330. 1	831	5.99/91.8	LOC10516661 0	LG7	18	III
SiHZ22b	XP_011084331. 1	805	6.08/88.6	LOC10516661 0	LG7	18	III
SiHZ22c	XP_011084333. 1	803	6.05/88.6	LOC10516661 0	LG7	18	III
SiHZ23	XP_011085207. 1	847	6.18/93.1	LOC10516726 4	LG8	18	III
SiHZ24a	XP_020552078. 1	696	6.22/77.2	LOC10516736 1	LG8	11	IV
SiHZ24b	XP_020552079. 1	691	6.31/76.7	LOC10516736 1	LG8	11	IV
SiHZ24c	XP_020552080. 1	694	6.14/77	LOC10516736 1	LG8	11	IV
SiHZ25	XP_020551655. 1	273	6.51/31.1	LOC10516766 6	LG8	3	I
SiHZ26	XP_011085824. 1	259	4.84/29.9	LOC10516770 6	LG8	4	I
SiHZ27	XP_020551334. 1	660	6.59/74.3	LOC10516781 4	LG8	11	IV
SiHZ28	XP_011085930. 1	698	6.63/78.8	LOC10516781 4	LG8	11	IV
SiHZ29	XP_011086174. 2	733	5.83/81	LOC10516797 4	LG8	13	IV
SiHZ30	XP_011086401. 2	340	5.34/38.4	LOC10516814 8	LG8	3	I
SiHZ31	XP_011086973. 1	727	5.95/79.9	LOC10516854 1	LG8	10	IV
SiHZ32	XP_011087214. 1	293	5.04/33.4	LOC10516876 5	LG8	3	I
SiHZ33	XP_011089294. 1	838	6.00/91.8	LOC10516876 5	LG9	18	III
SiHZ34	XP_011089912. 1	839	5.81/91.9	LOC10517072 1	LG9	18	III
SiHZ35	XP_011093009. 1	230	4.97/26.5	LOC10517306 0	LG11	2	I
SiHZ36a	XP_011093166. 1	835	5.86/91.2	LOC10517318 9	LG11	9	IV
SiHZ36b	XP_011093167. 1	834	5.86/91.1	LOC10517318 9	LG11	9	IV
SiHZ37	XP_011093277. 2	315	8.64/35.4	LOC10517328 0	LG11	4	II
SiHZ38	XP_011093390. 1	373	5.89/41.1	LOC10517337 6	LG11	4	II
SiHZ40a	XP_011096101. 1	726	5.66/79.6	LOC10517537 2	LG12	13	IV

SiHZ40b	XP_011096102. 1	726	5.66/79.6	LOC10517537 2	LG12	13	IV
SiHZ40c	XP_011096103. 1	726	5.66/79.6	LOC10517537 2	LG12	13	IV
SiHZ40d	XP_020554107. 1	726	5.66/79.6	LOC10517537 2	LG12	13	IV
SiHZ41a	XP_011096678. 1	852	5.85/93.4	LOC10517578 8	LG12	18	III
SiHZ41b	XP_020554026. 1	853	5.85/93.5	LOC10517578 8	LG12	18	III
SiHZ41c	XP_020554106. 1	726	5.66/79.6	LOC10517578 8	LG12	18	IV
SiHZ42	XP_011097532. 1	305	4.92/34.4	LOC10517643 3	LG13	3	I
SiHZ43	XP_011098601. 1	281	6.08/32.2	LOC10517723 0	LG15	3	I
SiHZ44	XP_011099420. 1	269	8.09/29.9	LOC10517785 2	LG15	3	II
SiHZ45	XP_011100030. 1	328	4.73/36.9	LOC10517828 0	LG15	3	I

Supplementary Table S1 List of identified HD-ZIP TFs in sesame by protein sequence homology (*A. thaliana*) and domain(s) assignment by HMM (Hidden Markov's Model)

Protein ID	Gene symbol	Linkage group (LG)	Chromosomal location	Exon count	SiHD-ZIP assigned name
1 XP_011075631.1	LOC105160061	LG1	9307691..9310369	4	SiHZ1
2 XP_011080516.1	LOC105163757	LG1	11862772..11869357, complement	18	SiHZ2
3 XP_011087051.1	LOC105168612	LG1	15178310..15180598	5	SiHZ3a
4 XP_011087055.1	LOC105168612	LG1	15178310..15180598	5	SiHZ3b
5 XP_011089804.1	LOC105170646	LG1	16504182..16506768, complement	4	SiHZ4
6 XP_011091721.2	LOC105172078	LG1	17471921..17477926	9	SiHZ5
7 XP_011070327.1	LOC105156012	LG2	12848062..12854969	13	SiHZ6a
8 XP_011070328.1	LOC105156012	LG2	12848062..12854969	13	SiHZ6b
9 XP_011070329.1	LOC105156012	LG2	12848062..12854969	13	SiHZ6c
1 XP_011070385.1	LOC105156051	LG2	13373296..13374667, complement	2	SiHZ7
1 XP_011070725.1	LOC105156327	LG2	15262273..15264616	4	SiHZ8
1 XP_011071017.1	LOC105156551	LG2	17326495..17328901	3	SiHZ9
1 XP_011071034.1	LOC105156564	LG2	17420046..17422085	3	SiHZ10
1 XP_011072039.1	LOC105157339	LG3	5902722..5906521	10	SiHZ11
1 XP_011072373.1	LOC105157647	LG3	8060164..8068299, complement	11	SiHZ12a
1 XP_020548412.1	LOC105157647	LG3	8060164..8068299, complement	11	SiHZ12b

1	XP_011072422.1	LOC105157678	LG3	8290214..8296829	18	SiHZ13
1	XP_011073660.1	LOC105158569	LG3	16324701..16326391, complement	4	SiHZ14a
1	XP_011073661.1	LOC105158569	LG3	16324701..16326391, complement	4	SiHZ14b
2	XP_011075258.1	LOC105159770	LG4	365616..367287, complement	4	SiHZ15
2	XP_011075470.1	LOC105159939	LG4	3530547..3532360, complement	3	SiHZ16
2	XP_020549042.1	LOC105160292	LG4	8756816..8758064	2	SiHZ17
2	XP_011076208.1	LOC105160506	LG4	10138345..10143621, complement	9	SiHZ18
2	XP_011076980.1	LOC105161099	LG4	15015420..15028495, complement	18	SiHZ19a
2	XP_011076981.1	LOC105161099	LG4	15015420..15028495, complement	18	SiHZ19b
2	XP_011076983.1	LOC105161099	LG4	15015420..15028495, complement	18	SiHZ19c
2	XP_011081291.1	LOC105164355	LG6	11199920..11201843	4	SiHZ20
2	XP_011081776.1	LOC105164733	LG6	17013344..17019333, complement	19	SiHZ21
2	XP_011084330.1	LOC105166610	LG7	8239690..8255794, complement	18	SiHZ22a
3	XP_011084331.1	LOC105166610	LG7	8239690..8255794, complement	18	SiHZ22b
3	XP_011084333.1	LOC105166610	LG7	8239690..8255794, complement	18	SiHZ22c
3	XP_011085207.1	LOC105167264	LG8	273987..280075	18	SiHZ23
3	XP_020552078.1	LOC105167361	LG8	1066413..1076252, complement	11	SiHZ24a
3	XP_020552079.1	LOC105167361	LG8	1066413..1076252, complement	11	SiHZ24b
3	XP_020552080.1	LOC105167361	LG8	1066413..1076252, complement	11	SiHZ24c
3	XP_020551655.1	LOC105167666	LG8	3612903..3614911	3	SiHZ25
3	XP_011085824.1	LOC105167706	LG8	3921244..3924299, complement	4	SiHZ26
3	XP_020551334.1	LOC105167814	LG8	6769979..6780673, complement	11	SiHZ27
3	XP_011085930.1	LOC105167814	LG8	6769979..6780673, complement	11	SiHZ28
4	XP_011086174.2	LOC105167974	LG8	8640252..8645949	13	SiHZ29
4	XP_011086401.2	LOC105168148	LG8	10039939..10041760	3	SiHZ30
4	XP_011086973.1	LOC105168541	LG8	13955207..13959698, complement	10	SiHZ31
4	XP_011087214.1	LOC105168765	LG8	14739116..14742059, complement	3	SiHZ32
4	XP_011089294.1	LOC105168765	LG9	2170825..2178059, complement	18	SiHZ33
4	XP_011089912.1	LOC105170721	LG9	5519865..5528848	18	SiHZ34
4	XP_011093009.1	LOC105173060	LG11	NC_026155.1 (194980..196348)	2	SiHZ35
4	XP_011093166.1	LOC105173189	LG11	1460439..1466477, complement	9	SiHZ36a

4	XP_011093167.1	LOC105173189	LG11	1460439..1466477, complement	9	SiHZ36b
4	XP_011093277.2	LOC105173280	LG11	2728727..2731873, complement	4	SiHZ37
5	XP_011093390.1	LOC105173376	LG11	5679695..5681389, complement	4	SiHZ38
5	XP_011096101.1	LOC105175372	LG12	3439532..3445476, complement	13	SiHZ40a
5	XP_011096102.1	LOC105175372	LG12	3439532..3445476, complement	13	SiHZ40b
5	XP_011096103.1	LOC105175372	LG12	3439532..3445476, complement	13	SiHZ40c
5	XP_020554107.1	LOC105175372	LG12	3439532..3445476, complement	13	SiHZ40d
5	XP_011096678.1	LOC105175788	LG12	5556645..5562726, complement	18	SiHZ41a
5	XP_020554026.1	LOC105175788	LG12	5556645..5562726, complement	18	SiHZ41b
5	XP_020554106.1	LOC105175788	LG12	5556645..5562726, complement	18	SiHZ41c
5	XP_011097532.1	LOC105176433	LG13	4332486..4335004	3	SiHZ42
5	XP_011098601.1	LOC105177230	LG15	820457..822331, complement	3	SiHZ43
6	XP_011099420.1	LOC105177852	LG15	4439230..4441002	3	SiHZ44
6	XP_011100030.1	LOC105178280	LG15	9118552..9121545	3	SiHZ45

Supplementary Table S2 Prediction of conserved domains (amino acid sequences) and their function(s) in SiHD-

Motif #	Conserved amino acid sequences	Width	Expasy Prosite predicted domain	InterPro predicted domain/Molecular function
1.	KKKRYHRHTPRQIQEMEAFFKECP HPDDKQRLSRELGLEPRQVKFW FQNRRTQMKAQHERHENSQRLAE NEKLRAENIR YREALRNAICPNCGGPA	98	Homeobox	Homeobox-leucine zipper protein/DNA binding site
2.	LGJQPRQVKVWFQNRRAREKQKQ EEVDLZ	29	Unidentified	None/DNA binding
3.	PGQPPGJVLSAATSFWLVPVKRVF DFLRDENSRSZWDILSNGGPVQEM AHIANGRDPGNCVSLRA	67	Unidentified	Homeobox-leucine zipper protein
4.	SKVTWVEHVELEASSVHEVYRPL YESGLAFGAKRWVAALDRQCZRL AETM	50	START	Homeobox-leucine zipper protein/Lipid binding
5.	PGNNNGAJZLMYAZLQVPSPLVPT REFYFLRYCKQHEDGSW	41	Unidentified	Homeobox-leucine zipper protein/Lipid binding
6.	ALPVFTFANQAGLDMLETTLVALQ DJTLEKILDGSGRKSLEFPTIMQQ GFAILPGGICLSSMGRPVSIEQAVV WKVLNE DGSVHC	87	Unidentified	None
7.	KGEASRESGVVIMNPIKLVEILMD VNKWSRVFRCIVSRAKT	41	Unidentified	None
8.	GEGRKSLKLZRMVRGFCDAVN GSTDDGWTTLSGDGADDV	41	Unidentified	Homeobox-leucine zipper protein/No Go term
9.	QSNMLILQESCTDSTGSYVVYAPV DIVAMNVVLSGGDPSYIALPSGF AILPD	53	Unidentified	Homeobox-leucine zipper protein/No Go term
10.	RKKLRLTKEQVEALEKSFEENKL NPKRK	29	Unidentified	None
11.	KPMIJELAVAAMDELIRMAQTGEP LWIPSTDNGAEVLNEEEYVRTFPR GI	50	Unidentified	Homeobox-leucine zipper protein/Lipid binding
12.	ZSLRAENARLKEELDRJKALV	21	Unidentified	None
13.	DJYLLQLCSGMDGTGVGACSELIF APIDRTFPBDAPLLPSGFRIPLDSN TGESTAS	57	Unidentified	None
14.	LKPLPGSPEAVTLARWICQSYRSH LGVELLGABCESTDVSLKQJWHHQ DAILCCSFK	57	Unidentified	None
15.	SVJCAKASMLLQNVPPALLVRFLR EHRSEWADYNVDDYSAAFLKAAP YAYPGPRIGNLSGTPV	63	Unidentified	None
16.	AASPRSLTJAFZFPFEFLKATVT AVARQYVRSVKSSVQS	41	Unidentified	None
17.	ASFVRCRRLPSGCLIQDMPNG	21	Unidentified	None/lipid binding
18.	DMGHKTPENEMDLIRDDEYESKS GSDIMEAPSGDDQDPNQR	41	Unidentified	None
19.	ANGAPZVGSSGSLTVAFQILVDSS PTAK	29	Unidentified	None
20.	QLPATTLTMCPSCERVATTT	20	Unidentified	None

ZIP proteins (Class I-IV)

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Supplementary Table S3 List of primers of SiHD-ZIP Class I genes

SiHD-ZIP Class I member genes	mRNA id	Forward primer	Reverse primer	Product length (bp)
SiHZ7	XM_01107208 3.2	TCTAAGCAAATCGAGCAA GAG	TCTGTCTACCTGGGTGAGC	114
SiHZ9	XM_01107271 5.2	CGATGGAAGACTAAGCAG TT	CTTCAGTGCCATTATCTCG G	129
SiHZ10	XM_01107273 2.2	GTGAAAGCTATGGAGGGTT G	CAGAGCCTGAAACATGAA AAG	148
SiHZ16	XM_01107716 8.2	TTCATGCTGAGATATTGGC AC	CTGGAGTCGTCGTTGAAAT A	138
SiHZ17	XM_02069338 3.1	TAAGAGTAGGGCTTTTCCA CA	ACTGTTTCCTCATCGTCTG T	165
SiHZ25	XM_02069599 6.1	GCTCCTCAAGACTTAAACG ATGT	TTCAAAGCTCCTCTCCAGG G	186
SiHZ26	XM_01108752 2.2	ATTGTGGTTGAGTGAGGGT T	TACTTCCACCTCGAAAAAT CG	148
SiHZ30	XM_01108809 9.2	TGTTCTCTGAGAATTTTCG CC	GATGCAGAGGCATGAAAA GAAG	181
SiHZ32	XM_01108891 2.2	GCTGGAAACAGATTACGA CA	CTTTTAGACGAAGAACCTC CG	113
SiHZ35	XM_01109470 7.2	CTTGGCTTCCCAGGTTGAA G	ATCCCCTTCCGGGCTATTT C	126
SiHZ42	XM_01109923 0.2	GAGAGGGATTATGGGGTTC T	GCTCTCCAATCTCTTTGAG T	103
SiHZ43	XM_01110029 9.2	TCAAACACAGTTATTGGCC C	GATGTTGTGTTGAGGTTGA C	132
SiHZ45	XM_01110172 8.2	TCTACAGATGAAAACAGCC C	CTCCTCTTCTTCTCCGAAA C	137

Fig. 1 Classification of HD-ZIP TF family in sesame - Maximum likelihood (ML) tree of 61 identified HD-ZIP transcription factors (TFs) from sesame. Full protein sequences of the HD-ZIP TF family in sesame were aligned by ClustalW and the ML tree was constructed by using MEGA (version X). Each class is labelled

Fig. 2 Maximum likelihood (ML) tree of 13 identified HD-ZIP Class I transcription factors (TF) in sesame along with *A. thaliana* and *Zea mays* HD-ZIP Class I TFs. Full protein sequences of HD-ZIP Class I from sesame, *A. thaliana* and *Zea mays* were aligned by ClustalW, and the ML tree was constructed using MEGA (version X). Names of subgroups are indicated (α , $\beta 1$, $\beta 2$, ζ , δ , ϵ , γ and φ)

Fig. 3 The conserved domain analysis of HD-ZIP Classes (I-IV) protein sequences using MEME. Different colors represent the different conserved motifs. The motif sequences are presented in Supplementary Table 2

Fig. 4 Domain(s) architecture of HD-ZIP Class I-IV in sesame, predicted by SMART represents (I) SiHZ7 and other SiHZ members proteins of Class I; (II) SiHZ1 and other SiHZ members proteins of Class II; (III) SiHZ2 and other SiHZ members proteins of Class III; (IV) SiHZ5 and other SiHZ members proteins of Class IV

Fig. 6 Effect of drought stress on sesame early seedling growth. The seedlings were >20 days old, grown at $\pm 25^{\circ}\text{C}$ (A) Control (well-watered) seedlings (B) Drought stress was imposed to the seedlings by water withholding for 7 days and soil moisture content reached up to ~16%. The experiment was performed in three replicates

Fig. 7 Expression analysis of 13 identified HD-ZIP Class I genes by qPCR. The actin gene was used as reference gene. Subgroups: Values are means and capped lines are standard errors (n = 3). *, Significant (P < 0.05) change relative to the control

Figures

Figure 1

Classification of HD-ZIP TF family in sesame - Maximum likelihood (ML) tree of 61 identified HD-ZIP transcription factors (TFs) from sesame. Full protein sequences of the HD-ZIP TF family in sesame were aligned by ClustalW and the ML tree was constructed by using MEGA (version X). Each class is labelled

Figure 2

Maximum likelihood (ML) tree of 13 identified HD-ZIP Class I transcription factors (TF) in sesame along with *A. thaliana* and *Zea mays* HD-ZIP Class I TFs. Full protein sequences of HD-ZIP Class I from sesame, *A. thaliana* and *Zea mays* were aligned by ClustalW, and the ML tree was constructed using MEGA (version X). Names of subgroups are indicated (α , β_2 , β_1 , γ , δ , ϵ , γ and ϕ)

Figure 4

Domain(s) architecture of HD-ZIP Class I-IV in sesame, predicted by SMART represents (I) SiHZ7 and other SiHZ members proteins of Class I; (II) SiHZ1 and other SiHZ members proteins of Class II; (III) SiHZ2 and other SiHZ members proteins of Class III; (IV) SiHZ5 and other SiHZ members proteins of Class IV

Figure 5

Gene structures of identified SiHD-ZIP Class I-IV by GSDS version 2.0

Figure 6

Effect of drought stress on sesame early seedling growth. The seedlings were >20 days old, grown at ± 25 °C (A) Control (well-watered) seedlings (B) Drought stress was imposed to the seedlings by water withholding for 7 days and soil moisture content reached up to ~16%. The experiment was performed in three replicates

Figure 7

Expression analysis of 13 identified HD-ZIP Class I genes by qPCR. The actin gene was used as reference gene. Subgroups: Values are means and capped lines are standard errors (n = 3). *, Significant (P < 0.05) change relative to the control

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