

Genome-wide analysis and gene expression studies revealed putative homeotic genes with a role in flower formation in sesame (*Sesamum indicum* L.)

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ABSTRACT

Sesame (*Sesamum indicum* L.) is an ancient oilseed crop with medicinal and nutritional value. Timely regulation of flowering could increase the sesame crop's seed productivity potential. To regulate flowering in sesame, it is necessary to identify the genes involved in flower development. Although several homeotic genes with a role in flower development have been identified in model plant species such as *Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia hybrida*, the homeotic genes in sesame need to be detected. It is hypothesized that a set of homeotic genes is conserved with a role in flower formation in sesame. The study aimed at identifying the homeotic genes through a genome-wide *in silico* search using the *Sesamum* genome database and gene expression studies. Our study revealed 23 putative homeotic genes that exhibited MADS domain, a characteristic of the homeotic genes, along with nine putative transcription factors with a role in flower formation. Furthermore, the gene expression studies revealed the five putative ABCDE class of genes –*SiAPI*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3*, respectively, as the critical players representing each of the five classes of ABCDE genes in sesame, confirming their function in floral induction and floral organ identity. The homeotic genes identified in this study could be explored further through gene manipulation and complementation studies to understand the mechanism of flowering in sesame.

1. INTRODUCTION

Sesame (*Sesamum indicum* L.) is an ancient oilseed crop, generally known as “queen of oilseeds” owing to its nutritional and medicinal value. It is a highly valued crop due to its oil content, which ranges from 41.3% to 62.7% [1], besides serving as a rich source of antioxidants and unsaturated fatty acids [2]. The sesame seeds, which are often used in culinary, confectionery, and medicinal purposes, are a rich source of oil that is highly resistant to oxidative deterioration [3]. Despite its economic importance, sesame is primarily grown as a marginal crop in arid and semi-arid regions worldwide [4]. Moreover, sesame, being a short-day plant, requires shorter day-length to promote flowering. Since flowering is a vital phenomenon that culminates in the seed-setting process, there is a possibility of enhancing the productivity potential of the sesame crop by regulating the flowering timing of those plants [5].

Flowering in angiosperms involves several synchronized events that are regulated by both genetic and environmental factors. While the genetic factors that regulate flower development comprise the homeotic genes, the environmental factors that influence flowering in plants include plant age, photoperiod, temperature vagaries, carbon-to-nitrogen ratio, and hormonal balance [6]. The interplay of these floral homeotic genes results in the establishment of distinct organ identities as well as their differentiation into distinct whorls, namely: sepals, petals, stamens, and carpels [7]. Although previous studies have reported the homeotic genes with a role in flowering in the model plant species, namely, *Arabidopsis thaliana* [8], *Antirrhinum majus* [9], and *Petunia hybrida* [10], the homeotic genes involved in flowering in sesame are yet to be identified. With this rationale, it is hypothesized that a similar set of homeotic genes might be involved in regulating floral induction and organ identity in sesame, and these homeotic genes might be conserved across the different plant members, including *S. indicum*.

Previous reports on the draft genome sequencing of the high oil-yielding (59%) Chinese sesame genotype, Zhongzhi No. 13, revealed an estimated number of 27,148 genes with the possibility of further genetic studies in sesame [11]. However, the functions of most of the sesame genes remain largely unknown, with only a few gene families been detected in sesame such as the MADS family of genes with a role

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in plant growth and development [12]. In the genome of *S. indicum*, 57 MADS-box genes were identified that showed predominant phylogenetic association with *A. thaliana*, *Utricularia gibba*, and *Solanum lycopersicum*, and were located to 14 linkage groups of *Sesamum* chromosomes. Although the MADS-box genes were identified in seven different tissues of sesame with a predicted role in growth and development, the relative expression of the homeotic genes at different stages of the flower and in different parts of the sesame flower needs to be explored. A recent study involving a genome-wide search for candidate MADS-box genes in wild and cultivated red gram (*Cajanus cajan* L.) revealed 71 of those genes to be evolutionarily conserved in three wild *Cajanus* species, namely, *C. cajanifolius*, *C. platycarpus*, and *C. scarabaeoides* [13]. Therefore, based on the above rationale, an attempt is made to understand the molecular mechanism that regulates floral induction and organ identity in sesame through genome-wide identification of homeotic genes using an *in silico* approach and gene expression studies, and the findings are presented.

2. MATERIALS AND METHODS

2.1. A Genome-wide Search for Putative Floral Homeotic Genes

The genome database of sesame (*S. indicum* L.) (tax id: 4128) available in the National Center for Biotechnology Information (NCBI) was utilized for putative floral homeotic genes. The reported sequences belonging to the MADS-box family in the model plants—*A. thaliana*, *A. majus*, and *P. hybrida* were searched by BLASTn in the sesame database program (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000512975.1/). The homologous gene sequences were aligned using Clustal Omega (European Bioinformatics Institute, Cambridge, UK). Gene sequences with nucleotide homology $\geq 80\%$ were selected. The biological function of these sequences was predicted using InterPro and BLASTP tools. The subcellular localization of these homologous sequences was predicted using the LOCALIZER tool (<https://localizer.csiro.au/>). The putative promoters were detected using the Promoter-2.0 tool (<https://services.healthtech.dtu.dk/services/Promoter-2.0/>).

2.2. Genetic Similarity Analysis and Mapping of Putative ABCDE Genes

The homologous sesame gene sequences with a homology of $\geq 75\%$ were identified through BLAST hits along with their accession identity (ID). Likewise, to detect interspecific conservancy of these putative homeotic genes, the level of homology of the sesame sequences with other related plant members, such as *A. thaliana*, *A. majus*, and *P. hybrida*, was determined using the BLAST search tool. The location of the putative homeotic genes on the chromosomes or linkage group, the cDNA sequences were used as query sequences for the BLASTN search against the *S. indicum* whole genome (Sinbase). The putative open reading frames (ORFs) were detected using the NCBI ORF finder tool. The BLASTP of NCBI was used to determine the functional domains of the putative floral homeotic genes.

2.3. Phylogenetic Analysis and Protein-interaction Network of Putative Homeotic Genes

The sesame floral homeotic gene sequences were aligned using ClustalW with *Arabidopsis*, *Antirrhinum*, and *Petunia* gene sequences. A phylogenetic tree was constructed using the maximum likelihood method in the MEGA12 software using a 1000-replicate bootstrap sampling test. The evolutionary relationship was assessed based on the number of synonymous and non-synonymous substitutions per site,

as adopted previously [14]. The gene clusters in the phylogenetic tree correspond to the distinct classes in the ABCDE model of flowering. The STRING database search tool was used for the retrieval of interacting putative homeotic genes (<https://string-db.org>). This computation tool allows the detection of functional interactions of proteins by integrating both known and predicted protein–protein interactions.

2.4. Plant Material, Growth Conditions, and Sampling

The seeds of the sesame genotype, Rajeshwari, used in the study were obtained from the Indian Institute of Oilseeds Research (IIOR) at Rajendranagar, Hyderabad, Telangana, India. The plants were grown in the plant culture facility, University of Hyderabad, under optimal plant growth conditions comprising a temperature range of 21–25°C/16–19°C (day/night, respectively). The floral buds were harvested at five distinct stages as described previously [15,16] and listed in Supplementary Table S1. Samples comprising different parts of the flower, namely: Sepals, petals, androecium, and gynoecium, were harvested at the fourth-bud stage. The same-aged floral buds, 3–4 in number per floral stage, were harvested separately. The samples were frozen in liquid nitrogen and stored at -80°C .

2.5. Nucleic Acid Isolation, cDNA Synthesis, and Primer Design

DNA was extracted from the floral and leaf samples by the CTAB method as described previously [17]. RNA was extracted using TRIzol reagent (Takara) as directed by the manufacturer. The cDNA synthesis was performed using 2 $\mu\text{g}/\mu\text{L}$ of total RNA pooled from three biological replicates per treatment using the Prime Script reverse transcriptase-polymerase chain reaction (RT-PCR) Kit (Takara) following the manufacturer's protocol. Primers were designed from conserved domain sequences of putative homeotic genes. The primers used for RT-PCR [Supplementary Table S2] and real-time quantitative (RT-q) PCR [Supplementary Table S3], along with annealing temperatures, are listed.

2.6. Genomic and RT-PCR for the Putative Homeotic Genes

Genomic polymerase chain reaction (PCR) and RT-PCRs were performed using gene-specific primers for all 32 putative homeotic genes. The optimized genomic PCR conditions were: denaturation at 95°C for 4 min, followed by 35 cycles each with a denaturation step at 94°C for 40 s, and annealing temp between 49–70°C specific to the primers at 45 s and an extension step at 72°C for 1 min followed by a final elongation step at 72°C for 10 min which was performed on an Arktik Thermal Cycler machine (ThermoFisher Scientific). The cDNA amplification was performed by RT-PCRs for different floral stages and leaf samples following the conditions: Denaturation at 94°C for 4 min, followed by 35 cycles each with a denaturation step at 94°C for 40 s, and annealing temp between 49–60°C specific to the primers at 45 s and an extension step at 72°C for 70 s followed by a final elongation step at 72°C for 7 min which was performed on an Arktik Thermal Cycler machine. PCR amplification efficiency and optimization conditions were standardized for all the genes. The amplified cDNA products were sequenced employing the sequencing services at Eurofins Genomics India Pvt. Ltd.

2.7. Confirming ABCDE Genes by Cloning, Sequencing, and Multiple Sequence Alignment

The amplified cDNA products of 5 putative gene sequences for each of the ABCDE classes were cloned into the pGMET Easy vector and sequenced. Using the ORF finder program (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), the ORFs were detected. The *in silico* tool,

BLASTP, was used to search conserved protein domains. Multiple sequence alignment was performed to identify highly conserved sequences using Clustal Omega 1.2.4.

2.8. Validating ABCDE Gene Candidates by RT-qPCR

A sample of cDNA (1 µg) was added to a final volume of 10 µl containing 5.2 µl SYBR Green Master Mix Reagent (Takara) and specific primers (3 pmol) were set for RT-qPCR with the following PCR program: 95°C for 2 min followed by 40 cycles of 95°C for 15 s, specific annealing temperature for a specific gene for 30 s and 72°C for 20 s. The RT-qPCR was analyzed in triplicate in a 96-well PCR plate (Applied Biosystems) on the real-time Eppendorf Master cycler with gene-specific primers for DAPs [Supplementary Table S3]. The *Actin*, *DNA-J*, and *Ubiquitin6 (UBQ6)* [16] gene primers were used as internal controls for the normalization of gene expression. The relative fold change in gene expression was calculated by $2^{-\Delta\Delta Ct}$ for all the genes in the treatments [18]. Statistical analysis was performed by plotting RT-qPCR data using SigmaPlot 11.0 and ANOVA to test the significance of variation using GraphPad Prism version 7.04 (La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

In higher eudicotyledonous plants, the identity of the floral organs has been reported to be specified by different classes of homeotic genes, and the majority of these homeotic genes belong to the MADS-box family, which encodes the transcription factors [19,20]. The floral quartet model proposed by Smaczniak *et al.* [21] suggested that the A- and E-class protein complexes determine the development of sepals in the first floral whorl. In the second whorl, the A-, B-, and E-class protein complexes specify petals. In the third whorl, the B-, C-, and E-class protein complexes determine the formation of stamens. Finally, in the fourth whorl, the C- and E-class protein complexes specify carpels. Although MADS-box genes were initially found to be major players in floral organ specification, subsequent studies revealed their involvement in the morphogenesis of plant organs throughout the plant's life cycle. The MADS-box gene family is one of the largest families found in higher plants, with a large number of duplication events that allowed functional divergence of the individual paralogs [21].

In a previous study involving a genome-wide detection and analysis of MADS-box genes, a total of 57 genes were identified from 14 linkage groups (LGs) in the sesame genome, including 33 type II and 24 type I MADS-box genes [12]. The sesame MADS-box genes were analyzed in seven different types of tissues, indicating their ABCDE functions. Furthermore, to gain a deeper understanding of the process of transition from the vegetative to the reproductive phase in sesame, it is necessary to identify and analyze the putative homeotic genes involved in floral induction and floral organ identity. The present study involves the detection of putative homeotic genes and their expression at different stages of the flower and in different parts of the sesame flower in comparison to leaf tissue.

3.1. Homology-based Identification, Isolation, and Sequencing of Putative Homeotic Genes

A total of 32 putative floral homeotic or floral organ identity gene sequences were detected in the genome of *S. indicum*, which showed high sequence homology with the reported homeotic genes belonging to the MADS-box family and other floral development protein domains in the model plants, such as *A. thaliana*, *A. majus*, and *P. hybrida*, as shown in Table 1. The gene ontology studies revealed

that out of the 32 putative homeotic genes, 23 genes exhibited MADS domains, whereas nine were predicted to be the putative transcription factors (TFs). Of these nine TFs, three genes each were related to the *FLORICAULA/LEAFY (FLO/LFY)* and F-box superfamily, respectively, two genes exhibited the homeodomain superfamily, and one gene exhibited the *APETALA2 (AP2)* superfamily. The three putative homeotic genes –*SiLEAFY (SiLFY)*–, *SiFLORICAULA (SiFLO)*–, and *SiABERRANT LEAF AND FLOWER (SiALF)*–like genes detected in sesame exhibited *FLO/LFY* MADS-box domain with DNA binding motifs that were plant-specific. These sequences showed a significant level of similarity to the TFs of *LFY* of *A. thaliana*, *FLO* of *A. majus*, and *ABERRANT LEAF AND FLOWER (ALF)* of *P. hybrida*, respectively [Table 1], indicating their evolutionary conservation in angiosperms. The TF *FLO/LFY* was first identified in *A. majus* [22], and subsequently in several other plant species with an established role in flowering, especially during the transition from vegetative to reproductive phase [23].

Likewise, two putative homeotic genes –*WUSCHEL (SiWUS)*– and *SiTERMINATA (SiTER)*–like genes detected in sesame exhibited homeodomain/MADS-box domain and showed similarity to the TFs–*WUS* of *A. thaliana* and *TER* of *P. hybrida*, indicating their putative regulatory function in sesame. The TF, *WUS* and its ortholog, *PhWUSCHEL* or *TER* in *Petunia* are central to the maintenance of the shoot apical meristem (SAM) for shoot and floral meristem identity [24]. Previous studies have demonstrated the role of the *WUS* gene in the structural and functional integrity of the floral organs in *Arabidopsis* [25]. Further, the TF, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SiSOC)*–like gene of sesame carrying the MADS domain, showed significant homology with the *SOC1* gene of *A. thaliana*. The TF, *SOC1*, functions as a flowering pathway integrator by integrating multiple flowering signals from various external and intrinsic developmental factors [26]. The remaining three putative TFs detected in sesame, namely: *SiFIMBRIATA (SiFIM)*–, *SiUNUSUAL FLORAL ORGANS (SiUFO)*–, and *SiDOUBLE TOP (SiDOT)*–like genes belonging to the F-box superfamily, were found to be homologous to *UNUSUAL FLORAL ORGANS (UFO)*, *FIMBRIATA (FIM)*, and *DOUBLE TOP (DOT)* genes of *A. thaliana*, *A. majus*, and *P. hybrida*, respectively, indicating conservancy of these homeotic TFs across the plant species with a role in flowering. The TF, *UFO*, was the first plant F-box gene shown to function in floral meristem identity by activating *APETALA3* and *PISTILLATA*, which are required to establish the whorled pattern of floral organs in *Arabidopsis* [27].

Similarly, the remaining 22 genes with MADS domains identified in sesame in this study were homologous to various classes of *ABCDE* genes in *A. thaliana*, *A. majus*, and *P. hybrid*, as shown in Table 1. The 22 genes with MADS domain detected in the study were grouped into various classes of *ABCDE* genes, as previously identified by Thiessen [28]. This indicates that the MADS-box gene structure, expression, and function play a crucial role in plant reproductive development, involving flower, fruit, and seed formation. These putative gene sequences were grouped into separate classes: Class A with four gene homologs (*SiAPETALA1 [SiAP1]*, *SiPETUNIA FLOWERING GENE [SiPFG]*, *SiAPETALA2 [SiAP2]* and *SiSQUAMOSA [SiSQUA]*), Class B with seven genes (*SiAPETALA3 [SiAP3]*, *SiDEFECIENS [SiDEF]*, *SiTOMATO MADS-BOX GENE 6 [SiTM]*, *SiPISTILLATA [SiPI]*, *SiGLOBOSA [SiGLO]*, *SiGLOBOSA1 [SiGLO1]* and *SiMADS2*), Class C with four genes (*SiAGAMOUS [SiAGA]*, *SiPLENA [SiPLE]*, *SiMADS,3* and *SiFLORAL BINDING PROTEIN 6 [SiFBP6]*), and Class D with three genes (*SiSEED STICK [SiSTK]*, *SiFLORAL BINDING PROTEIN 7 [SiFBP]*, and *SiFLORAL*

Table 1: Putative floral homeotic genes detected in sesame.

Class	Putative homeotic genes in <i>Sesamum indicum</i>	Gene homologs identified in other plant spp.			Conserved domain/Function
		Arabidopsis	Antirrhinum	Petunia	
*TF	<i>SiLEAFY</i> (<i>SiLFY</i>), <i>SiFLORICAULA</i> (<i>SiFLO</i>) <i>SiABERRANT LEAF AND FLOWER</i> (<i>SiALF</i>)	<i>LEAFY</i>	<i>FLORICAULA</i>	<i>ALF</i>	DNA binding: plant-specific (FLO-LFY SP)/MADS BOX Domain
*TF	<i>SiWUSCHEL</i> (<i>SiWUS</i>) <i>SiTERMINATA</i> (<i>SiTER</i>)	WUS		TER	Homeodomain/MADS BOX Domain
*TF	<i>SiUNUSUAL FLORAL ORGANS</i> (<i>SiUFO</i>) <i>SiFIMBRIATA</i> (<i>SiFIM</i>) <i>SiDOUBLE TOP</i> (<i>SiDOT</i>)	<i>UFO</i>	<i>FIMBRIATA</i>	<i>DOT</i>	F-box Superfamily
*TF	<i>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1</i> (<i>SiSOC1</i>)	<i>SOC1</i>			MADS Domain
A	<i>SiAPETALA1</i> (<i>SiAP1</i>) <i>SiSQUAMOSA</i> (<i>SiSQUA</i>) <i>SiPETUNIA FLOWERING GENE</i> (<i>SiPFG</i>)	<i>APETALA1</i>	<i>SQUAMOSA</i>	<i>PFG</i>	MADS Domain
A	<i>SiAPETALA2</i> (<i>SiAP2</i>)				AP2 Domain
B	<i>SiAPETALA3</i> (<i>SiAP3</i>) <i>SiDEFICIENS</i> (<i>SiDEF</i>) <i>SiTOMATO MADS-BOX GENE 6</i> (<i>SiTM6</i>)	<i>APETALA3</i>	<i>DEFICIENS</i>	<i>TM6</i>	MADS Domain
B	<i>SiPISTILLATA</i> (<i>SiPI</i>) <i>SiGLOBOSA</i> (<i>SiGLO</i>) <i>SiGLOBOSA1</i> (<i>SiGLO1</i>) <i>SiMADS2</i>	<i>PISTILATA</i>	<i>GLOBOSA</i>	<i>GLO1</i> <i>MADS2</i>	MADS Domain
C	<i>SiAGAMOUS</i> (<i>SiAGA</i>) <i>SiPLENA</i> (<i>SiPLE</i>) <i>SiMADS3</i> <i>SiFLORAL BINDING PROTEIN 6</i> (<i>SiFBP6</i>)	<i>AGAMOUS</i>	<i>PLENA</i>	<i>MADS3</i> <i>FBP6</i>	MADS Domain
D	<i>SiSEED STICK</i> (<i>SiSTK</i>) <i>SiFLORAL BINDING PROTEIN 7</i> (<i>SiFBP7</i>) <i>SiFLORAL BINDING PROTEIN 11</i> (<i>SiFBP11</i>)	<i>STK</i>		<i>FBP7</i> <i>FBP11</i>	MADS Domain
E	<i>SiSEPELLATA</i> (<i>SiSEP1</i>) <i>SiSEPELLATA</i> (<i>SiSEP2</i>) <i>SiSEPELLATA</i> (<i>SiSEP3</i>) <i>SiDEFH49</i> <i>SiFLORAL BINDING PROTEIN 2</i> (<i>SiFBP2</i>)	<i>SEP1</i> <i>SEP2</i> <i>SEP3</i>	<i>DEFH49</i>	<i>SiFBP2</i>	MADS Domain

BINDING PROTEIN 11 [*SiFBP11*]). The five genes homologous to Class E genes were *SiSEPELLATA* (*SiSEP1*), *SiSEPELLATA* (*SiSEP2*), *SiSEPELLATA* (*SiSEP3*), *SiDEFH49*, and *SiFLORAL BINDING PROTEIN 2* (*SiFBP2*). Previous studies also revealed the detection of *SQUAMOSA* (*SQUA*) representing Class A, *DEFICIENS* (*DEF*)- or *GLOBOSA* (*GLO*) representing Class B, *AGAMOUS* (*AG*)- representing Class C and D, and *SEPALLATA* (*SEP*)-like genes representing Class E [28]. Our studies also revealed the conservancy of these genes in sesame with a role in floral organ identity.

The study also revealed the molecular characteristics of the putative homeotic genes detected in *S. indicum*, as shown in Table 2. The putative floral homeotic genes were categorized into *ABCDE* gene classes in sesame based on their conserved domains and amino acid sequences. Of the 32 gene sequences, 28 gene sequences were

identified in the *S. indicum* genome that showed homology to the previously reported genes in other plant species. Whereas the three genes: *SiSOC*, *SiTM6*, and *SiSTK*, whose gene sequences have been detected in this study based on *in silico* and gene expression studies, have not been annotated so far. These genes showed evolutionary conservancy with the *ABCDE* genes detected previously in other plant members, indicating their role in floral induction and flower development as previously described [19]. Further, these gene sequences were analyzed for linkage mapping, cellular localization, and promoter detection as shown in Table 2.

The cDNA sequences of all 32 putative homeotic genes were analyzed to identify protein domains and nucleotide similarity with those of the reported homeotic genes from other model plant species. The cDNA sequences of all 32 putative homeotic genes were submitted to the NCBI,

Table 2: Characteristic features of 32 putative floral homeotic gene sequences identified in sesame.

Gene Name	Gene		Gene sequence	mRNA sequence (bp)	Coding sequence (bp)	Protein sequence (aa)	Linkage group	Cellular-detected	Promoter detected
	Start	End							
<i>SiLFY</i>	13,606,522	13,617,947	11426	1764	1223	407	LG3	Nucleus	P
<i>SiFLO</i>	13,606,522	13,617,947	11426	1764	1223	407	LG3	Nucleus	P
<i>SiALF</i>	13606522	13617947	11426	1764	1223	407	LG3	Nucleus	P
<i>SiWUS</i>	9234035	9236969	2934	2029	1059	352	LG1	Nucleus	P
<i>SiTER</i>	13,606,522	13,617,947	11426	1764	1223	407	LG3	Nucleus	P
<i>SiUFO</i>	6202015	6205620	3606	3606	1322	440	LG12	Chloroplast	P
<i>SiFIM</i>	6202015	6205620	3606	3606	1322	440	LG12	Chloroplast	P
<i>SiDOT</i>	23434240	23436190	1951	798	705	234	LG3	Nucleus	P
<i>SiSOC1</i>	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>SiAP1</i>	16802802	16816066	13265	1917	1383	460	LG2	Nucleus	P
<i>SiSQUA</i>	10709817	10713094	3278	2431	1392	463	LG1	Nucleus	P
<i>SiPFG</i>	13738216	13747543	9328	1547	774	257	LG8	Mitochondria,	P
<i>SiAP2</i>	509396	512889	3494	1920	1416	471	LG5	Nucleus Nucleus	P
<i>SiAP3</i>	23434240	23436190	1951	798	705	234	LG3	Nucleus	P
<i>SiDEF</i>	23434240	23436190	1951	798	705	234	LG3	Nucleus	P
<i>SiTM6</i>	N/A	N/A	N/A	N/A	N/A	N/A	NA	N/A	N/A
<i>SiPI</i>	17831557	17833792	1956	919	639	212	LG6	Nucleus	NP
<i>SiGLO</i>	17831557	17833792	2,236	919	639	212	LG6	Nucleus	NP
<i>SiGLO1</i>	17831557	17833792	2,236	919	639	212	LG6	Nucleus	NP
<i>SiMADS2</i>	17833537	17833688	2,236	919	639	212	LG6	Nucleus	NP
<i>SiAG</i>	2,855,020	2,865,915	10,896	1059	696	231	LG14	Nucleus	P
<i>SiPLE</i>	23,106,254	23,114,208	7,955	1248	753	250	LG6	Nucleus	P
<i>SiMADS3</i>	12922	18268	5,347	1257	732	243	LG8	Nucleus	P
<i>SiFBP6</i>	23106254	23114208	7955	1248	753	250	LG6	Nucleus	P
<i>SiSTK</i>	NA	NA	NA	NA	NA	NA	NA	NA	N/A
<i>SiFBP7</i>	423532	428653	4801	1094	669	222	LG2	Nucleus	P
<i>SiFBP11</i>	423532	428653	4801	1094	669	222	LG2	Nucleus	P
<i>SiSEP1</i>	2339457	2344824	5368	1522	744	247	LG15	Nucleus	P
<i>SiSEP2</i>	2339457	2344824	5368	1522	744	247	LG15	Nucleus	P
<i>SiSEP3</i>	12847465	12856220	8756	1194	680	242	LG8	Nucleus	P
<i>SiDEFH49</i>	2,339,457	2,344,824	5,368	1522	744	247	LG15	Nucleus	P
<i>SiFBP2</i>	5,738	10,719	4,982	1201	714	237	LG8	Nucleus	NP

and the GenBank accession numbers are listed in Table 3. The nucleotide similarity and protein domain conservancy analyses revealed high sequence homology with other model plant species – *A. thaliana*, *A. majus*, and *P. hybrida*. The percentage nucleotide similarity ranged between 81% and 100%. The MADS-Box domains were found in the majority of the gene sequences, with the exception of the *SiAP2* (*SiAPETALA2*)-like gene, which is a member of the AP2 superfamily. The *APETALA2* gene differs from the other organ identity genes with regard to the absence of a MADS domain and a region-specific pattern [29].

3.2. Genetic-relatedness of Sesame Homeotic Genes with Orthologs from Other Plant spp.

To analyze the evolutionary relatedness of the detected homeotic genes of sesame with the reported floral organ genes identified from the model plants – *A. thaliana*, *A. majus*, and *P. hybrida*, a phylogenetic tree was constructed with the cDNA sequences of

67 homeotic genes, including the 32 potential genes from sesame detected in this study [Figure 1]. The study revealed homology-based grouping of all sesame homeotic gene sequences with their orthologous gene sequences from *A. thaliana*, *A. majus*, and *P. hybrida*. The sesame homeotic gene sequences were functionally categorized into five classes of the ABCDE model of flower development. These clusters comprised four gene homologs grouped as Class A, seven as Class B, four as Class C, three as Class D, five as Class E, and nine as the putative TFs, as discussed in section 3.1 of results and discussion, indicating their potential role in floral gene regulation. Previous studies also revealed that most organ identity genes are regulated at the mRNA level, possibly through direct transcriptional control. The floral homeotic control genes appear to encode transcription factors [28].

3.3. Identification of Protein Interaction Networks

Table 3: The partial cDNA sequences of the 32 putative homeotic genes in sesame obtained through RT-PCR-based gene expression, along with their accession numbers, domains, and nucleotide similarity with reported homeotic genes from other model plant species.

Gene Name	Partial cDNA sequence (bp)	Domain	Query sequences (Accession No.)	Petunia (%)	Antirrhinum (%)	Arabidopsis (%)
<i>SiLFY</i>	620	FLO-LFY SP	OQ092330	92	89	95
<i>SiFLO</i>	997	FLO-LFY SP	OQ092333	85	87	90
<i>SiALF</i>	635	FLO-LFY SP	OQ092335	86	83	89
<i>SiWUS</i>	232	Homeodomain	OQ092336	91	93	87
<i>SiTER</i>	625	Homeodomain	OQ092332	86	97	97
<i>SiUFO</i>	313	F-box	OQ092337	91	89	82
<i>SiFIM</i>	372	F-box	OQ092334	89	96	94
<i>SiDOT</i>	995	F-box	OQ092353	93	85	93
<i>SiSOC1</i>	962	MADS	OQ092331	80	86	96
<i>SiAP1</i>	790	MADS	KM677186	99	98	100
<i>SiSQUA</i>	606	MADS	OQ092352	92	99	97
<i>SiPFG</i>	382	MADS	OQ092338	86	92	93
<i>SiAP2</i>	883	AP2	KM190074	91	94	98
<i>SiAP3</i>	798	Superfamily MADS	KM190075	93	95	100
<i>SiDEF</i>	986	MADS	OQ033392	82	91	90
<i>SiTM6</i>	846	MADS	OQ092344	89	90	82
<i>SiPI</i>	680	MADS	OQ092350	96	83	91
<i>SiGLO</i>	288	MADS	OQ092349	93	89	85
<i>SiGLO1</i>	590	MADS	OQ092340	96	98	89
<i>SiMADS2</i>	352	MADS	OQ092348	82	96	92
<i>SiAG</i>	735	MADS	KM190076	98	99	100
<i>SiPLE</i>	576	MADS	OQ092347	92	95	90
<i>SiMADS3</i>	718	MADS	OQ092421	99	89	86
<i>SiFBP6</i>	420	MADS	OQ092343	97	94	83
<i>SiSTK</i>	579	MADS	OQ198411	95	90	99
<i>SiFBP7</i>	364	MADS	OQ092342	83	86	81
<i>SiFBP11</i>	372	MADS	OQ092345	93	96	82
<i>SiSEP1</i>	391	MADS	OQ092352	98	96	97
<i>SiSEP2</i>	691	MADS	OQ092351	92	93	96
<i>SiSEP3</i>	629	MADS	KF601336	100	99	100
<i>SiDEFH49</i>	596	MADS	OQ092346	97	89	88
<i>SiFBP2</i>	501	MADS	OQ092339	94	96	90

RT-PCR: Reverse transcriptase-polymerase chain reaction

Using the STRING tool, potential interactions between the reported floral proteins were detected with nodes corresponding to the proteins and the edges (lines) indicating the interactions. A total of 16 proteins out of 32 exhibited a significant level of interaction with each other, forming a network with a specific number of nodes. There are 16 edges in total. The *P*-value with $<1.0e-16$ for the enrichment of 81 protein-protein interactions suggested a significant level of protein-protein interactions. The TF, *SiLF*, showed strong correlation with several other proteins, namely: *SiAPETALA1* (*SiAP1*), *SiAPETALA3* (*SiAP3*), *SiAGAMOUS* (*SiAGA*), *SiPISTILLATA* (*SiPI*), *SiSEPALLATA1* (*SiSEP1*), *SiSEPALLATA2* (*SiSEP2*), and *SiSEPALLATA3* (*SiSEP3*). A homeotic gene, *GmAPI*, which encodes an *APETALA1*-like protein, was identified and isolated in soybean with transactivation activity and caused early

flowering along with alteration of floral organs, when ectopically expressed in tobacco plants [30]. Similarly mutational studies performed to analyze the interactions of a homeotic gene, *SHORT VEGETATIVE PHASE* (*SVP*) in *A. thaliana* showed that *SVP* binds to the promoters of *APETALA1*, *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *SEPALLATA3* (*SEP3*), as well the intron of *AGAMOUS* (*AG*) to regulate the expression of B-class and C-class floral homeotic genes, thereby modulating floral organ formation [8]. On the contrary, *SiWUS*, *SiAP2*, and *SiUFO* proteins exhibited weak associations, and their interaction was limited to a few proteins, as shown in Figure 2. The findings suggested that the majority of the homeotic genes analyzed in the study represented the downstream pathway genes of floral meristem identity in sesame.

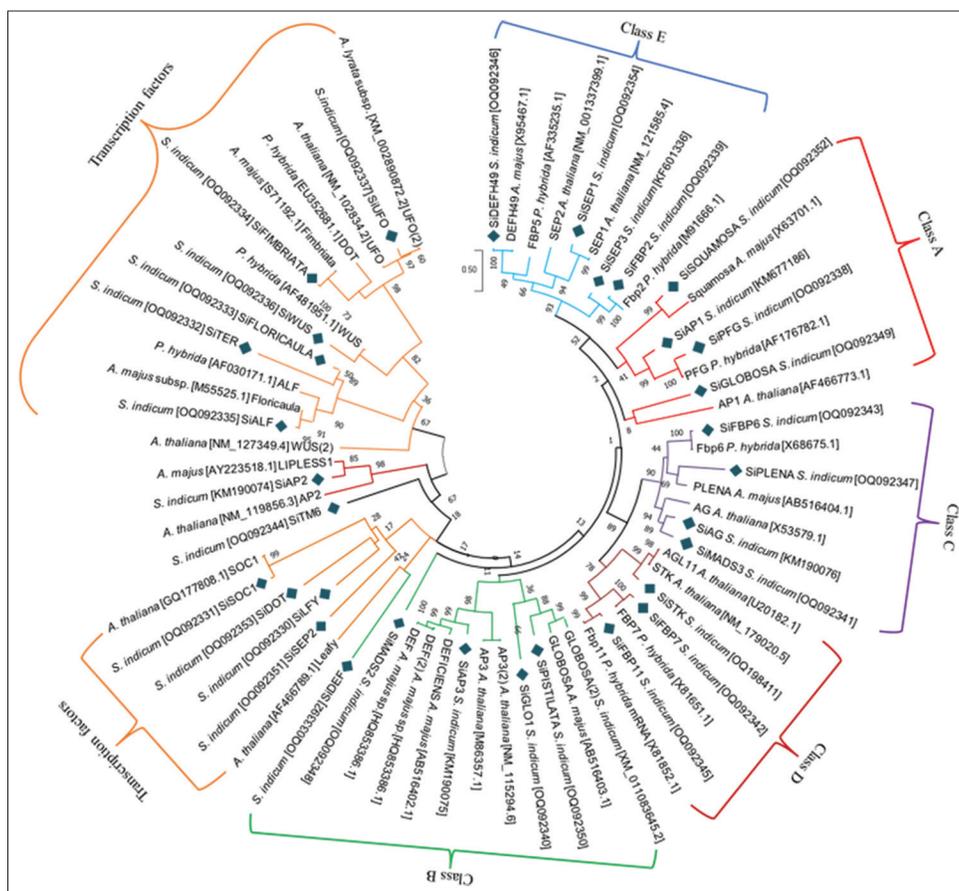


Figure 1: Phylogenetic tree drawn using putative sesame homeotic gene sequences in comparison to reported floral homeotic genes of other model plant species. A phylogenetic tree was constructed using the maximum likelihood method in the MEGA12 software using a 1000-replicate bootstrap sampling test. The 67 partial cDNA sequences of *Sesamum indicum*, *Arabidopsis thaliana*, *Petunia hybrida*, and *Antirrhinum majus* are indicated by green, pink, light blue, and dark blue colors, respectively.

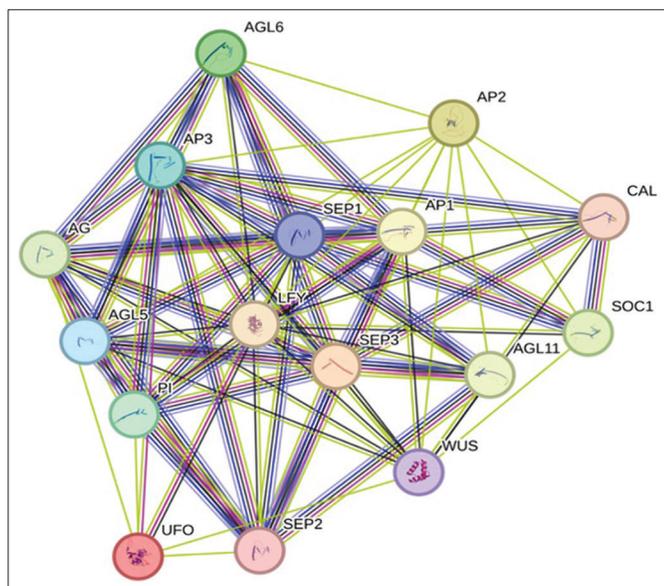


Figure 2: Protein-protein interaction networks of the 32 putative floral homeotic genes deciphered by the STRING online tool. Network nodes (bold circles) represent proteins. Edges (lines) show protein-protein association. The black coloured lines indicate actual interaction between proteins connecting one node to another. PPI enrichment with $P: <1.0e-16$.

3.4. Genomic PCR and RT-PCR Studies of the Putative Homeotic Genes

Genomic PCR was performed to monitor the presence of the 32 putative homeotic genes in sesame floral tissue. All 32 putative homeotic genes were confirmed through amplification of the expected band size of the genomic PCR products, as shown in Supplementary Figure S1.

Gene expression analysis of putative floral homeotic genes in sesame that are homologous to *ABCDE* gene classes and TFs of *A. thaliana*, by RT-PCR, revealed differential expression of these genes at different stages of flower formation, indicating their role in flowering [Supplementary Figure S2]. A total of nine putative homeotic genes and four putative TFs homologous to *A. thaliana* were expressed in sesame, suggesting the conservancy of these sequences in sesame in regulating flower formation. The mRNA transcripts of the A-class genes – *APETALA1* and *APETALA2*, along with the Class C gene, *AGAMOUS*, accumulated at higher levels in the floral buds, while their expression was negligible in the leaf sample. The homozygous mutants of the *APETALA* gene in *Arabidopsis* produced flowers that lack petals, and the loss of the petal phenotype was attributed to the failure of petal primordia development. Further, it was demonstrated that *APETALA1* and *APETALA2* genes, in concert with the protein product of the *AGAMOUS* gene, as detected in our study, were responsible for the determinate floral meristem structure and floral meristem pattern [31].

A similar pattern of gene expression was observed for the homologous sequences of the putative homeotic genes and TFs of *A. majus*

[Supplementary Figure S3] and *P. hybrida* [Supplementary Figure S4] when tested in sesame floral and leaf tissues. Five putative homeotic genes (*SiSQUA*, *SiDEF*, *SiGLO*, *SiPE*, and *SiDEFH49*) and two putative TFs (*SiFLO* and *SiFIM*) homologous to *A. majus* were found to be expressed in sesame also. The *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) genes reported in the model organism *A. majus* were reported as orthologous to *LEAFY* (*LFY*) and *APETALA1* (*API*) in *A. thaliana* [32]. The homeotic genes *SiDEF*, *SiGLO*, *SiPE*, and *SiPLE* previously reported in *A. majus* are found to be orthologous to the B-class genes – *APETALA3* and *PISTILATA*, and the C-class gene, *AGAMOUS* of *A. thaliana* [33]. In our study, the orthologs of these genes were identified in sesame, indicating similar redundancy of these genes as a requirement for maintaining floral organ identity in sesame. Likewise, eight putative homeotic genes and three putative TFs homologous to *P. hybrida* were expressed in sesame, suggesting their conservancy across plant species [Supplementary Figure S4]. Three TFs, *SiALF*, *SiDOT*, and *SITER*, which were previously identified as floral meristem identity (FMI) genes with spatiotemporal control of floral identity expression patterns [34]. The majority of these genes are differentially expressed in floral tissues, while their expression was at a basal level in leaf tissues, suggesting their role in floral induction and flower development.

3.5. Cloning and Sequence Characterization of *SiAP1*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3*

The cDNA products amplified by the gene-specific primers of the selected five putative floral organ identity genes – *SiAP1*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3*, representing the five gene classes – A, B, C, D, and E, respectively, of the flower development model were cloned, sequenced, and submitted to GenBank. Using the BLASTP *in silico* tool, the conserved domains and amino acid residues were deciphered for these potential sesame homeotic genes. The ClustalW analysis of these sesame homeotic gene sequences showed significant homology with the reported homeotic genes, confirming them as the ABCDE class genes.

3.6. Gene Expression by RT-qPCR

The RT-qPCRs were carried out to analyze the expression patterns of the putative homeotic genes and floral organ identity gene sequences representing all five classes of ABCDE flower model – *SiAP1*, *SiAP3*,

SiAG, *SiSTK*, and *SiSEP3* at different stages of floral buds [Figure 3a] and in different parts of the flower (stage-4) [Figure 3b] presented graphically in comparison to the expression in leaf tissue in sesame.

3.6.1. Putative floral organ identity gene expression at different stages of flower

A distinct pattern of expression was observed for each of the five homeotic genes at different stages (stages 1–5) in comparison to leaf tissue, as shown in Figure 3a. The expression of all five homeotic genes: *SiAP1*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3* was at a basal level in leaf tissue when compared to the floral stages, indicating their role in floral organ identity. Gene expression of the putative Class A gene, *SiAP1*, though expressed at all stages of the flower, its expression was higher at stages 4 and 5, with 2 and >2 log-fold change gene expression, respectively, indicating its role in the formation of the fully developed floral organs at later stages. This finding suggested the requirement of *SiAPETALA1* throughout the floral stages, in agreement with the previous studies demonstrating *APETALA1*'s functioning in primarily repressing vegetative identity and establishing floral primordia formation, followed by differentiation of the floral parts [35].

Similarly, the B-class gene *SiAP3* increased considerably from stage 1 to stage 5, with 2.4- to 4.0-fold-change expression levels. Similar spatial and temporal variations were detected in *APETALA3* gene expression at different stages of flower development, as it controls the formation of petals and stamens during flower development [36]. The Class C gene, *SiAG*, showed a substantial increase between stages 1–3, with a 2.3- to 3.3-fold change in expression, whereas stages 4 and 5 showed consistency in their mRNA abundance levels. This could be due to its functioning as a principal developmental switch to regulate the transition from indeterminate to determinate floral growth [37]. The Class D gene, *SiSTK*, showed an increase in the expression levels at later stages of flower formation between the stages, 3 to 5, compared to the early stages of 1 and 2, indicating its function in the formation of carpels in a flower, in agreement with the earlier findings of the regulatory function of *STK* in ovule integument identity [38]. Of all the genes, the Class E gene *SiSEP3* showed low expression levels at the juvenile phase (stages 1 to 3) with a slight increase at the mature phase from 2 log fold at stage 4 to <2 log fold change at stage 5 of floral bud formation. The expression of the *SEP3* gene was found to be specific to floral organs, with a role

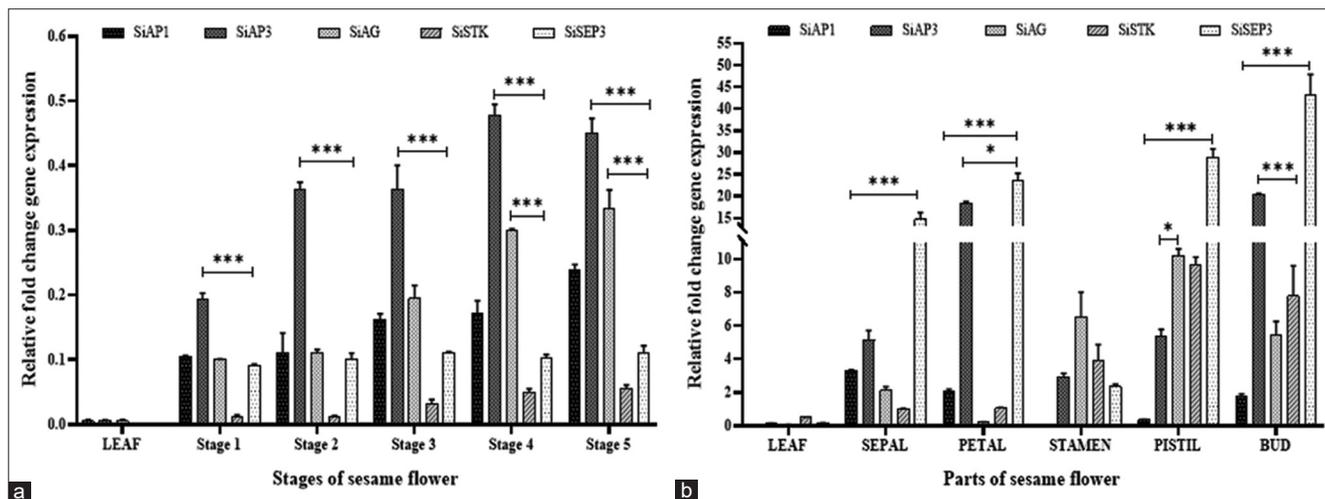


Figure 3: The real-time quantitative polymerase chain reaction analysis of the five putative sesame homeotic genes and floral organ identity gene sequences – *SiAP1*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3* representing the five classes of ABCDE model at (a) different stages of flower formation and (b) different parts of the flower (stage-4). Expression in intact floral bud and leaf is shown as controls. The mRNA expression was set as 2 log-fold as calculated by the $2^{-\Delta\Delta C_T}$ method, normalized with the mRNA expression of the *Ubiquitin6* gene (*UBQ6*). Non-template DNA control (NTC) was used as a negative control. Red color: High significance level; blue color: Low significance level.

in specifying different floral whorls, as well as promoting flowering and regulating flowering timing in plants, as demonstrated by the ectopic expression of *Phalaenopsis PeSEP3* in *Arabidopsis* [39].

3.6.2. Putative floral organ identity gene expression in different whorls of the sesame flower

To investigate the expression patterns of the putative homeotic genes – *SiAPI*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3* in different parts of the flower, a comparative gene expression study was carried out in all four whorls – sepals, petals, stamens, and carpels, along with intact floral bud and leaf tissue. The RT-qPCRs for the five homeotic genes, though they revealed differential gene expression among the different floral organs, their expression was at a negligible level in leaf tissue, as shown in Figure 3b. The mRNA transcripts of the *SiAPI* gene were higher in sepals and floral buds, whereas *SiAPETALA3* was expressed predominantly in petals and floral buds, typical with their role in sepal and petal formation [19]. On the contrary, the *SiAGAMOUS* gene is expressed in stamens, carpels, and intact floral buds. Whereas the *SiSTK* gene was found to be expressed in carpels and floral bud alone, the *SiSEP3* mRNA transcripts accumulated in all the whorls in increasing order of sepals, petals, stamens, carpels, and the floral bud in sesame, as anticipated in accordance with the previous findings revealing the ABC model of genes controlling the floral organ identity in plants [19,20]. Studies on these genetic components and their interactions could be further explored to effectively employ the genes involved in flowering in breeding programs, to alter the phenology of sesame plants to produce new varieties with improved flowering and reproductive attributes compared to traditional cultivars.

4. CONCLUSION

Despite the importance of sesame as an essential oilseed crop, it is primarily grown in rainfed environmental conditions worldwide, leading to poor production. The productivity potential of sesame could be enhanced by genetic modulation of the flowering time and duration of flowering. Our understanding of the molecular mechanism underlying floral initiation and development in sesame is limited. Therefore, the present study was undertaken to identify putative homeotic genes involved in flower formation in sesame. Based on the sequence homology of the reported homeotic genes in the model plant species such as *A. thaliana*, *A. majus*, and *P. hybrida*, the putative homeotic genes in sesame were identified, and their expression patterns were analyzed. Five homeotic genes – *SiAPI*, *SiAP3*, *SiAG*, *SiSTK*, and *SiSEP3* were cloned and analyzed for their expression at different stages of the flower and in different parts of the flower. These genes were identified as the *ABCDE* genes of sesame, and they could be further explored through genetic manipulation and transformation studies to confirm the mechanism of flowering in sesame.

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in

drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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The authors declare that they have not used artificial intelligence (AI) tools for writing and editing of the manuscript, and no images were manipulated using AI.

13. SUPPLEMENTARY MATERIAL:

The supplementary material can be accessed at the journal's website: Link here [https://jabonline.in/admin/php/uploadss/1434_pdf.pdf]

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