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Expression profiling and molecular characterization of *PhFBP26* MIKC type MADS box TF gene during floral developmental stages of *Petunia hybrida*

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Abstract
Petunia hybrida, well known as bedding plant belongs to solanaceae family of plants and widely utilized as research model plant in scientific community. The plant MIKC type MADS box genes, activate or repress the expression of their target genes based on the nature of their cofactors which majorly affect all developmental process of plant life cycle. Here, we reported the molecular characterization, phylogenetic association and expression profile of *PhFBP26 MIKC* TF gene during floral transition and flower developmental stages from *P. hybrida.* In phylogenetic study, we found the association of *PhFBP26* with *AtAGL8* and *FUL2* gene in Arabidopsis and tomato respectively. Further, the expression pattern of *PhFBP26* was recorded in all 4 floral developmental stages along with petal, sepal and leaf tissue of petunia which, may suggest its conserved involvement during floral transitions pathways in plants.

Keywords: MADS box, FBP, Motifs, in-silico, petunia hybrida, garden petunia

1. Introduction
Garden petunia (*Petunia hybrida*), is the most common bedding plant in the world for its variety of color and morphology has both annual and perennial cultivars belonging to the solanaceae family which also includes other species that are significant from an agronomic standpoint, such as *S. Tuberosum*, *L. Esculentum*, *Capsicum Annuum*, and *N. Tabacum.* The genus Petunia, which Jussieu first described in 1803, has its roots in South America and is regarded as the first domesticated bedding plant. It has remained one of the most popular genera for the creation of new variations (Vandenbussche, *et al.*, 2016) [17]. The petunia is widely used as a model system to study the evolution of floral organs, the transition of the vegetative meristem into the reproductive organ, the florescence and development of buds, the activity of transposons, the self-incompatibility of genes, and interactions with microbes, herbivores and pollinators. Bombarely, *et al.*, 2016 [3] reported the complete genome sequencing of P. *Axillaris N* and *P. Inflata S6* (parents of *P. Hybrida*) and assembly include 91.3% and 90.2% coverage of their diploid genomes (1.4 Gb; 2n = 14) containing 32,928 and 36,697 protein-coding genes, respectively.

During their lifespan, plants go through a number of developmental phases, each of which is marked by the expression of unique morphological features and/or the emergence of new organs. One such developmental shift in angiosperms is from the vegetative growth phase to the reproductive growth phase for the formation of the flowers. Different routes control the proper timing of floral transition switches (from vegetative to flowering), which is essential for plants' reproductive success (Waheed & Zeng 2020) [18]. For further advancements in agricultural practice, a thorough comprehension of the regulatory mechanisms determining flowering time is crucial due to the fact that flowering time has a substantial impact on both plant fitness and crop output (Hong & Jackson., 2015) [5].

The widely used ABC-model of flower development, originally proposed by Coen and Meyerowitz in 1991, initially comprised three classes of functional genes, namely, MADS box protein-related genes A, B, and C, which specify the domains of the floral whorls of developing flowers. Later, the model was expanded to include D and E functions based on petunia studies (Ferrario, *et al.*, 2004) [4]. The presence of a highly conserved 60-amino-acid DNA-binding domain, which is involved in binding to DNA based on a consensus CC(A/T)6GG (also known as the CArG box) sequence, can be used to identify the MADS-Box genes, which are known as the architects of flower formation (Sasaki, *et al.*, 2010) [13].

The acronym MADS domain was derived from four members of the family *viz*. Mini Chromosome Maintenance1 (MCM1) from Saccharomyces cerevisiae (Passmore, *et al.*, 1998) [10], Agamous (AG) from Arabidopsis (Yanofsky, *et al.*, 1990) [20], Deficiens (DEF) from Antirrhinum majus (Sommer, *et al.*, 1990) and Serum Response Factor (SRF) from Homo sapiens (Norman, *et al.*, 1998) [8].

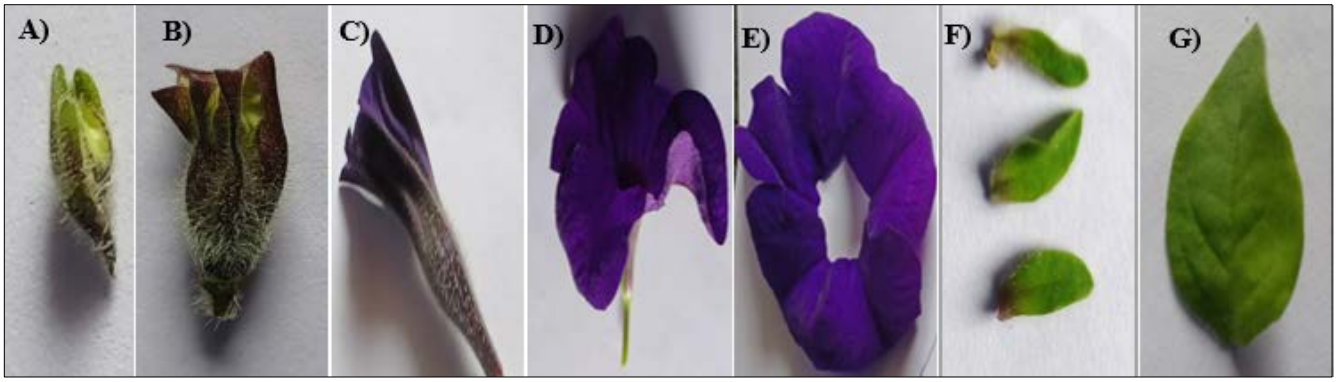
On the basis of the evolutionary relationship of the conserved MADS box domain, the MADS-box genes family was further classified into the two primary types: type I and type II genes. Plant type I MADS-domain genes are referred to as M-type genes and further divided into three groups: Mα, Mβ and Mγ. Type II MADS-domain genes are referred to as MIKC-type, and they are composed of the highly conserved MADS-domain (M), a moderately conserved intervening (I) domain, a well-conserved keratin-like (K) domain, and a highly divergent carboxy terminal (C) domain and classified into two subgroups $MIKC^{C}$ and MIKC* based on their structural characteristics (Smaczniak, *et al.*, 2012) [14]. During the evolution of angiosperms, the MADS-box genes underwent diversification, which has influenced the diversity of floral development (Abraham *et al.*, 2020) [2]. Additionally, MIKC-type genes are involved in the formation of inflorescences and the timing of flowering and the response of roots to nitrate deficiency (Zhou, *et al.*, 2022; Qu, *et al.*, 2021) [21, 11].

In present study, we molecularly characterized and recorded the expression profile of *PhFBP26* MIKC type MADS box TF gene from different floral developmental stages to assess the role of this gene during floral transition in *Petunia hybrida*. The aim of current investigation is to maximize the understanding of *PhFBP26 MIKC* type *MADS* box genes and function.

2. Materials and Methods

2.1 Plant Materials and sample collection

The seeds of *Petunia hybrida* (Blue) were collected from Department of Horticulture, SVPUA&T, Modipuram, Meerut and grown under favorable growth conditions. Here, 4 different flower growth stages (i.e., Initial flower bud [A], mature bud [B], unopened flower [C], opened flower [D]), only petal [E], only sepal [F] and leaf [G] of *Petunia hybrida* were used as samples (Figure 2.1) for total RNA isolation.



A-Initial bud, B-Mature Bud, C-1 Days before Opening Flower, D-Opened Flower, E-Only Mature Petals, F- Sepals Only, G- Petunia Leaf

Fig 2.1: Different growth stages of petunia flower and leaf sample

2.2 Molecular characterization and expression analysis of *PhFBP26* MIKC type MADS Box TF of *P. Hybrida*

2.2.1 Data mining and primer designing

The publicly available *FBP26* gene sequence of *P. hybrida* was downloaded from the NCBI database (ftp://ftp.ncbi.nih.gov/blast/db/) to design the forward and reverse gene specific primers. The primers were designed manually and statistically verified using Oligo-Calc. (http://biotools.nubic.northwestern.edu/OligoCalc.html).

2.2.2 Isolation and expression analysis of *PhFBP26* TF gene

The total RNA was isolated from 100mg fresh petunia samples (as mentioned in section 2.1 & Fig.1) using Pure Link™ RNA Mini kit (Invitrogen, Life Technologies), then reverse transcribed into cDNA utilizing Oligo-dt as primers using ImProm-II™ Reverse Transcription System (Promega) as per the manufacturer's protocol. The coding region of *PhFBP26* gene was PCR amplified (with the Promega components except dNTPs from Thermo-scientific) using synthesized cDNA as template using gene specific primers by initial denaturation at 94°C for 3 minutes followed by 35 cycles of 1 minute of denaturation at 94 °C, 45 seconds of annealing at 57°C followed by extension of 1 minute at 72 °C and final extension of 10 minutes at 72 °C in an Eppendorf Master cycler PCR machine. To screen the presence of *PhFBP26* TFs mRNA transcripts, expression analysis was carried out using the synthesized cDNA from all the floral developmental stages and selected tissues (petals, sepals and leaves) of *P. hybrida* with similar PCR conditions as above described. The PCR amplified product was then subjected to 1% agarose gel electrophoresis and visualized on a UV transilluminator and documented using a gel documentation system (UviTec Cambridge, England).

2.2.3 Cloning, Sequencing and evolutionary analysis

The specific amplicon of *PhFBP26* was purified from gel using NucleoSpin® Gel and PCR clean up kit by Macherey-Nagel with described user's manual. The purified PCR amplicons derived from *PhFBP26* gene was cloned separately in pGEM-T Easy cloning vector (3015bp, Promega, USA) with optimal molar ratio of 1:3 vector to insert proportion. The ligated product was mobilized into competent cells of *E. coli* strain DH5α with heat and shock method and the recombinant clones were initially selected on the basis of Blue/White screening followed by confirmation with colony PCR and restriction digestion-based assay. The positive recombinant plasmid was sequenced and the resulting assembled and molecularly characterized *PhFBP26* gene sequence was submitted to NCBI public domain. The newly

sequenced nucleotides were subjected to BLAST analysis using NCBI server (http://www.ncbi.nlm.nih.gov/blast/) followed by phylogenetic analysis with *MIKC* Type MADS Box sequence of *A. thaliana* and *S. lycopersicum* using the MEGA version 11.0 (Tamura, *et al.*, 2021) [16].

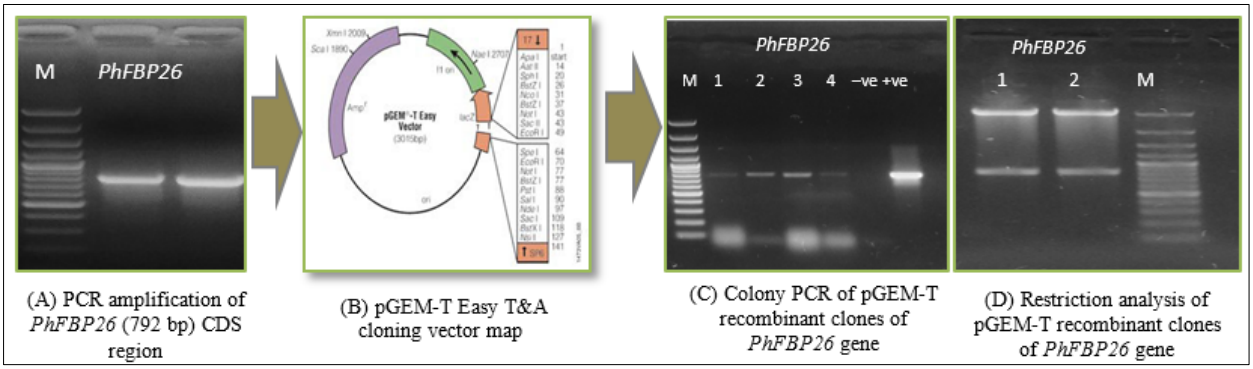
2.3. Prediction of regulatory TFs and Gene Ontology analysis of *FBP26* genes of petunia

The binding sites for TFs (also called regulatory TFs) were predicted for *PhFBP26* MIKC genes by treating their 1.5kb upstream DNA region as promoter region using Plant Transcriptional Regulatory Map, a plant regulatory data and a nalysis platform (http://plantregmap.gao-lab.org/network.php) which help to advancing the understanding of plant regulatory system. The Gene Ontology (GO) enrichment for *FBP26* was performed based on inbuilt topGO (v2.22) and Fisher's exact tests in Plant Transcriptional Regulatory Map tool system.

3. Results and Discussions

3.1 Molecular cloning, sequencing and evolutionary study of *PhFBP26:* The complete gene fragment of *PhFBP26* (~ 792 bp) was PCR amplified from flower bud cDNA using gene specific forward and reverse primers i.e., *PhFBP26-F*'- ATG GGG AGA GGA AGA GTG C and *PhFBP26-R*' AGC ATA ATA CTG ACC GCT TGC (Figure 3.1)**.** The PCR product was cloned into pGEM-T cloning vector followed by the transformation of recombinant plasmid into DH5α (*E. coli* cells) competent cells. Further, the confirmed recombinant plasmid was sequenced and generated sequence was checked for BLAST analysis and submitted to NCBI database (under releasing process). Further, to know the structural variation, the coding DNA sequences of *PhFBP26* was considered as query sequences for comparative analysis with their respective gene sequences as previously reported in *P. hybrida, P. Axillaris & P. Inflata*. Here, we found that the new gene sequence of *PhFBP26* was found to be different at 15 Nt positions with old sequence *PhFBP26* (> AF176783.1) followed by 14 Nt differences with *PeaxiFBP26* and 11 Nt differences with *PeinFBP26.* Similarly, the differences in Aa sequences were also reported for query *PhFBP26* Aa and found to dissimilar at two positions with old *PhFBP26*, single position with *PeaxiFBP26* and three Aa differences in *PeinFBP26* (Figure. 3. 2 A & B).

The gene sequences of *PhFBP26* were searched to find their evolutionary closes *MIKC*-Type *MADS* Box gene in Arabidopsis (*Arabidopsis thaliana*, 39) and Tomato (*Solanum lycopersicum*, 32) using Mega program v. 1000 bootstrap replicates in Neighbor joining (NJ) phylogenetic analysis. In the phylogenetic study, the gene sequence PhFBP26 was evolutionary associated with AtAGL8 (SQUAMOSA subfamily) and FUL2 (Fruit full) TF (Solyc03g114830.2.1, AtAGL8 homolog). The multiple sequence analysis of PhFBP26 protein sequence also revealed the presence of MIKC domains (Figure 3.4) and phylogenetic study revealed its association with AP1/SQUA subfamily of MIKC family genes.



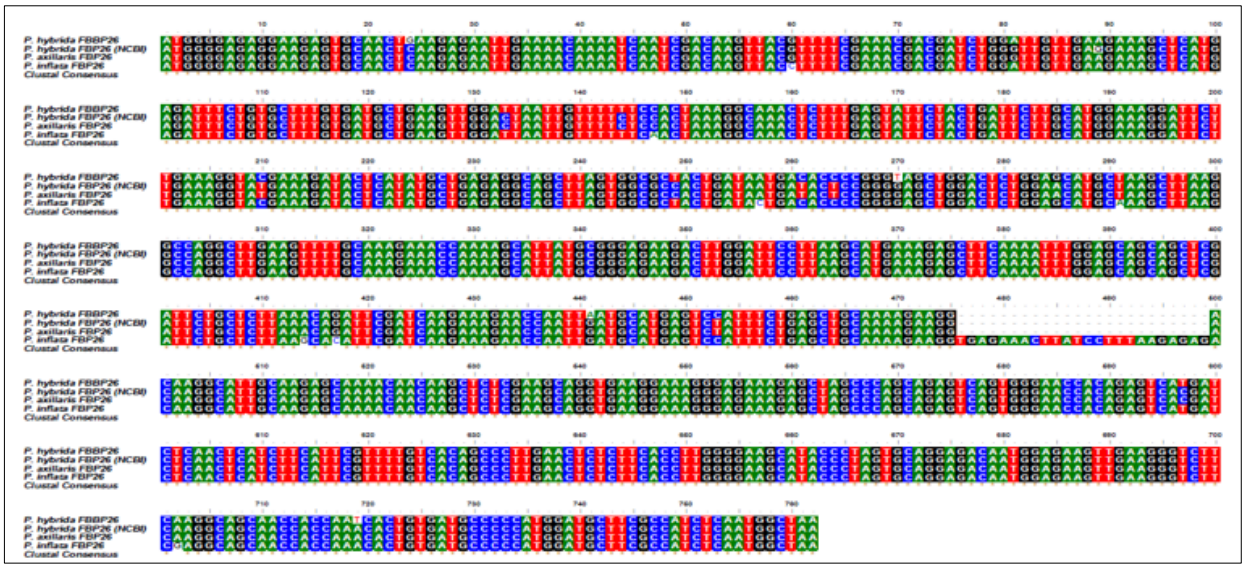
(A) PCR amplification of *PhFBP26* (792 bp) CDS region

(B) pGEM-T Easy T&A cloning vector map

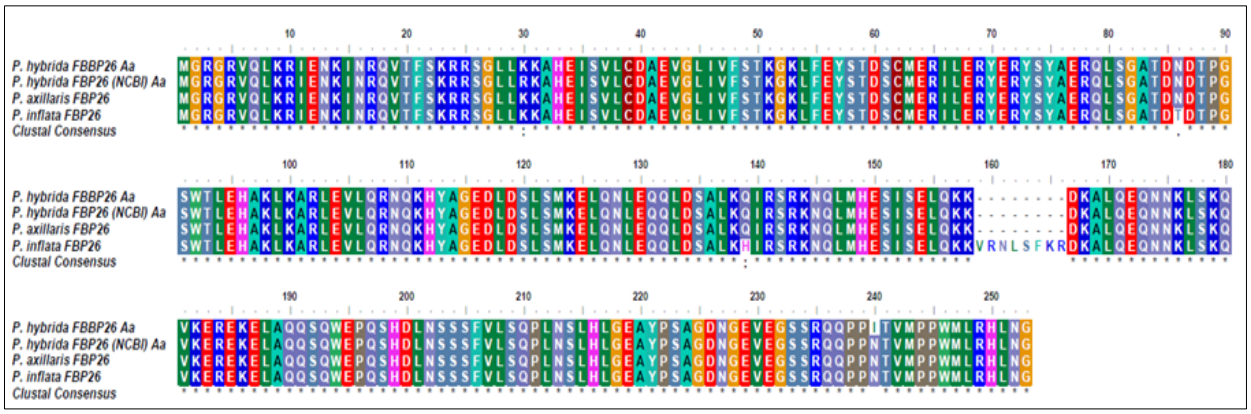
(C) Colony PCR of pGEM-T recombinant clones of *PhFBP26* gene

(D) Restriction analysis of pGEM-T recombinant clones of *PhFBP26* gene

Fig 3.1: Schematic representation of the PhFBP26 PCR amplification, cloning in pGEM-T Easy T&A cloning vector & recombinant confirmation



(A)



(B)

Fig 3.2 A & B: Comparative view of multiple sequence alignment of *PhFBP26* with *FBP26* TF gene from *P. Axillaris & P. Inflata* along with previously reported *PhFBP26* in NCBI.

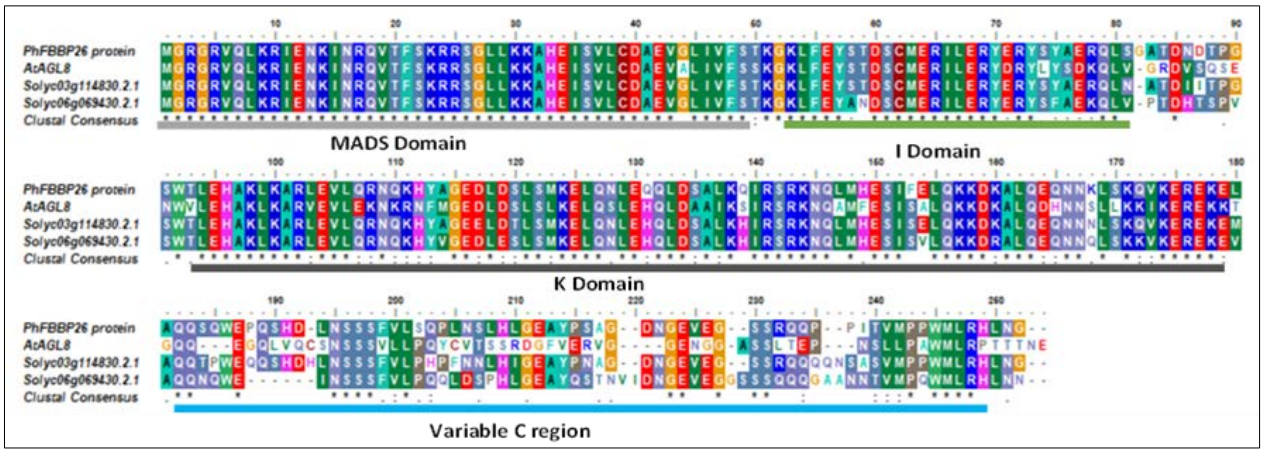


Fig 3.3: Multiple sequence alignment of *PhFBP26* amino acid sequence with phylogenetically close genes in *A. thaliana (AtAGL8)* & *S. lycopersicum (Solyc03g114830.2.1* and *Solyc06g069430.2.1) MIKC-type MADS-Box* using Bio Edit software version 5.0.9. The regions of the MADS domain, I domain, K domain and the C region are underlined.

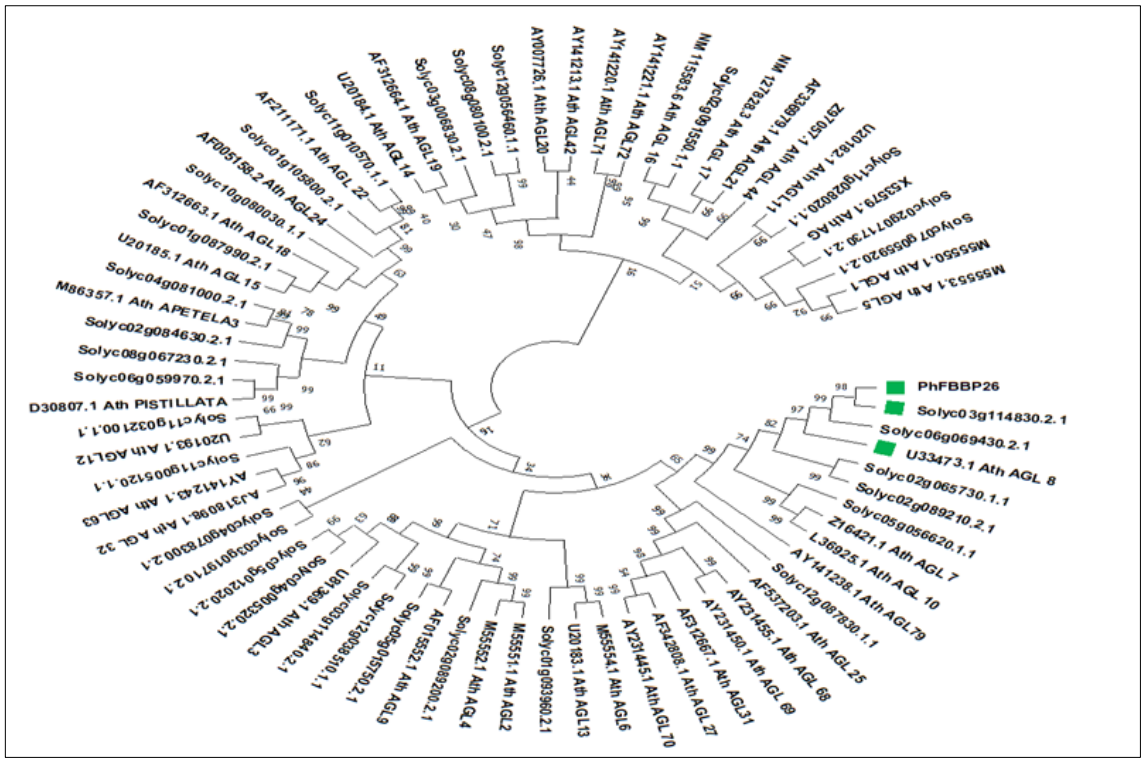
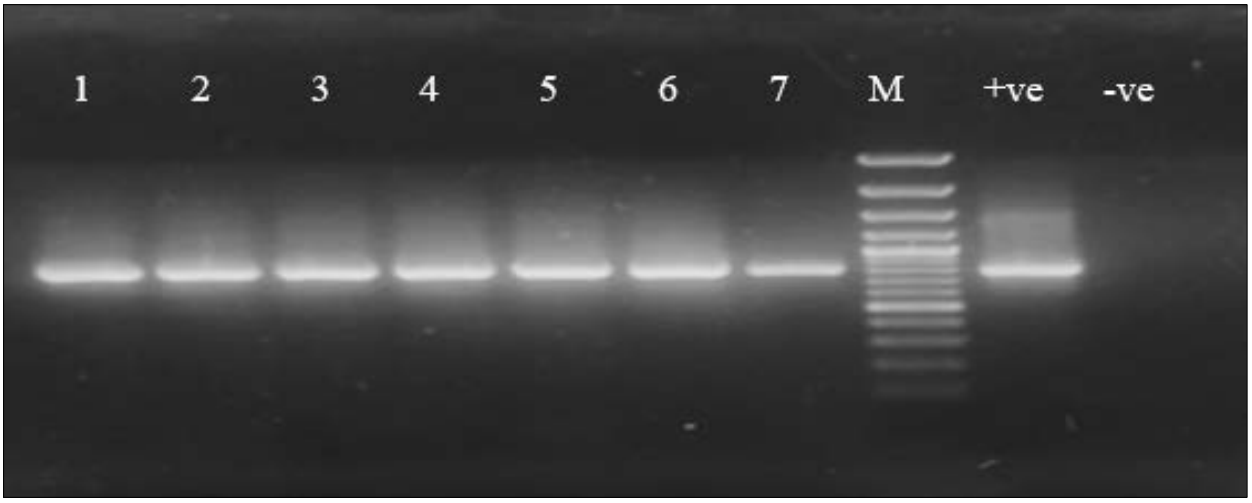


Fig 3.4: Phylogeny analysis of nucleotide sequences of *PhFBP26* gene with *MIKC* Type *MADS* Box genes of *A. thaliana & S. lycopersicum* using Neighbour-joining method in Mega v.11.0 software

3.2 Expression analysis of *PhFBP26 MIKC* type MADS box gene

The expression pattern of *PhFBP26* TF gene was investigated at 4 different developmental stages of flower (reproductive part), petal, sepals exclusively and leaf (vegetative part) from *P. hybrida* using synthesized cDNA of respective sample. The *PhFBP26* transcripts were recorded to express with a constant rate in both the vegetative and reproductive samples of petunia hybrida (Figure 3.5). Consequently, it could be suggested that *PhFBP26* has role in floral transition pathways and all floral growth stages of petunia flowers.



Expression analysis of *PhFBP26* from reproductive and vegetative parts of *P. hybrida*

3.3 Regulatory TFs and Gene Ontology analysis of *PhFBP26* genes

The regulatory elements and interactions between them, advancing the understanding of plant transcriptional regulatory system. The regulatory TF proteins were predicted for *FBP26* (Peaxi162Scf00017g03265) based on binding capacity of other TFs to its promoter region. Here, we found a total of 14 regulatory TFs for *FBP26* gene of petunia, among them maximum were annotated as Ethylene-Responsive-Element-Binding Factor (Table 3.1).

Table 3.1: List of regulatory TFs of FBP26 MIKC type MADS box gene of petunia

Sr. No	Regulatory TFs I.D	Description from PlantTFDB
1	Peaxi162Scf00006g00229	NAC Family Protein
2	Peaxi162Scf00009g00023	Dof Family Protein
3	Peaxi162Scf00016g00183	Ethylene Responsive Element Binding Factor 1
4	Peaxi162Scf00031g00144	*P. hybrida* Ethylene-Responsive-Element-Binding Factor 6
5	Peaxi162Scf00038g02229	*P. hybrida* Ethylene-Responsive-Element-Binding Factor 2
6	Peaxi162Scf00065g01120	*P. hybrida* Ethylene-Responsive-Element-Binding Factor 5
7	Peaxi162Scf00271g00061	Ethylene Responsive Element Binding Factor 1
8	Peaxi162Scf00420g00827	B3 Family Protein
9	Peaxi162Scf00461g00112	Ethylene-Responsive Transcription Factor 12
10	Peaxi162Scf00569g00053	Ethylene-Responsive Transcription Factor ABR1-Like
11	Peaxi162Scf00778g00021	Dof Family Protein
12	Peaxi162Scf00832g00315	Dof Family Protein
13	Peaxi162Scf00993g00011	Dof Family Protein
14	Peaxi162Scf01066g00013	Ethylene-Responsive Transcription Factor 4

The Gene Ontology (GO) provides a rigorously defined set of concepts that describe the functions of gene products and represented as an association between a gene product and the biological concept. The GO terms are associated with gene products based on two general approaches: from experimental data and from sequence inferences. So, the 7 Gene Ontology (GO) terms were assigned to *FBP26* (Peaxi162scf00020g 02337.1) which, involved in Regulation of transcription, DNA-template (GO: 0006355), Transcription factor activity, sequence-specific DNA binding (GO:0003700), Nucleus (GO:0005634), Protein dimerization activity (GO:0046983), DNA binding (GO:0003677), fruit development (GO:0010154) and Maintenance of inflorescence meristem identity (GO:0010077).

As previously reported, that MADS-box gene family is involved in nearly every aspect of plant growth and development, it is essential to identify and define its members (Othman *et al.*, 2016) [9]. Importantly, it is essential to know the primary gene structures, functional domains, protein structural motifs, and protein structures of the MADS-box genes in order to understand the flowering pathways in plants. Additionally, phylogenetic comparative methods (PCMs) are crucial for resolving a variety of concerns regarding the evolution of species. Researchers can investigate how traits change over time, the order in which related traits evolve, and how ecology affects a trait's advancement using phylogenetic relationships. Previously, in order to characterize the roles of the *SEPALLATA3* MADS box TF complexes at the molecular level, Kaufmann, *et al.*, 2009 analyzed genome-wide DNA-

binding patterns using chromatin immune precipitation followed by ultrahigh-throughput sequencing and revealed that *SEPALLATA3* binds to thousands of sites in the genome and characterized target genes integrates and modulates different growth-related and hormonal pathways in a combinatorial fashion with other MADS-box proteins and possibly with non-MADS transcription factors. Moreover, Ren, *et al.*, 2021 [12] previously, identified and characterized 43 non-redundant MADS-box genes from the flower organs transcriptase's of *A. amurensis* to understand flowering time regulation and floral organogenesis and revealed that the ABCDE model genes were highly expressed mainly in flowers and differentially expressed in the different tissues of flower organs, suggesting that they may be involved in the flower organ identity of *A. amurensis*. The identification and expression profile of 45 *MIKC-type MADS*-box genes was also reported by Qu, *et al.*, 2021 [11] and revealed that most of the were expressed in six tissues (specifically expressed in floral buds), and visibly varied in the same subfamily in *Cyclocarya paliurus*. Similarly, Wang, *et al.*, 2022 also found that ABCDE genes were specifically expressed in floral buds or petals, consistent with their function of regulating flower organ development in rose plant. Further, the evolutionary analysis and expression pattern of *MIKC Type MADS Box* genes suggest that this family of TFs play significant role during plant growth and flower development.

4. Conclusions

In plants, the MIKC-Type MADS-Box genes play major role in flower development from the early step of determining the identity of floral meristems to specifying the identity of floral organ primordia later in flower development in addition to physiological and developmental processes of plants. The current investigation of expression and molecular characterization of *PhFBP26* may be utilize to understand their role in floral transition-related pathways and flower development in petunia plants. The present information may contribute in further studies to improve flowering traits through novel genetic engineering approaches in flowering plants.

5. Acknowledgments

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