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Cloning and Expression Analysis of The FaesSEP4_3 Gene From Fagopyrum Esculentun

Zhuofan Chen
College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China

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Abstract

To explore the function of SEP4 homologous genes in the flower and fruit development of lpls common buckwheat, a SEP4 homologous cDNA sequence with a full length of 916 bp was isolated from lpls common buckwheat using the homologous cloning method. This gene contains a complete open reading frame (ORF) of 690 bp, encoding 229 amino acids and a stop codon. Phylogenetic analysis showed that it belongs to the SEP4 lineage of the SEP subfamily within the MADS-box transcription factor family, and it was named FaesSEP4_3. Protein sequence alignment indicated that FaesSEP4_3 contains a highly conserved MADS domain composed of 62 amino acid residues and a secondary conserved K domain composed of 80 amino acid residues. Meanwhile, its C-terminal domain contains two conserved motifs, SEPI motif and SEPII motif. Tissue expression analysis of the FaesSEP4_3 gene in lpls common buckwheat via real-time quantitative PCR (qRT-PCR) revealed that FaesSEP4_3 is mainly expressed in roots, tepals, stamens, pistils, and fruits. Moreover, expression levels of FaesSEP4_3 in tepals, stamens, pistils, and fruits was obviously higher than that of the roots. In addition, FaesSEP4_3 is obviously expressed throughout all stages of flower bud development. These data suggests that FaesSEP4_3 gene may be involved in floral organ and fruit development of common buckwheat.

Keywords

Buckwheat; Flower Development; SEPALLATA4; MADS-Box; Gene Expression

1. Introduction

Common buckwheat (Fagopyrum esculentum) is a non-cereal annual herbaceous plant belonging to the genus Fagopyrum in the family Polygonaceae, order Caryophyllales. It is also an economic crop with multiple uses, including food, medicine, ornamental value, and soil remediation. It features a short growth cycle, strong stress resistance, and high tolerance to cold and barren conditions [1-2]. The grains of common buckwheat are not only rich in nutrients such as proteins and cellulose but also possess pharmacological effects like antioxidant activity, blood pressure reduction, and decreased capillary fragility, thus holding high nutritional

value and health benefits [3-4]. However, as a typical distylous crop, the natural population of common buckwheat segregates into short-styled with long stamens (thrum type) and long-styled with short stamens (pin type) plants in a 1:1 ratio [5-6]. This forms a heteromorphic self-incompatibility (HSI) system, where normal seed setting occurs only through inter-morph pollination (between pin and thrum types), while intra-morph pollination fails to set seeds. This leads to low seed setting rates under natural conditions, severely restricting the healthy development of the common buckwheat industry [7-8].

Long pistil and long stamen (lpls) common buckwheat, characterized by stable genetic traits, good compatibility, and the ability to set seeds normally through self-pollination as well as pollination between homostylous and heterostylous flowers, serves as a precious germplasm resource for high-yield breeding of common buckwheat. In-depth research on the developmental patterns and molecular regulatory mechanisms of flowers and fruits in lpls common buckwheat is the prerequisite and foundation for the rational utilization of this material.

In the model plant Arabidopsis thaliana, the SEPALLATA4 (SEP4) gene is mainly involved in the whorled arrangement of sepal, spetals, stamens, and carpels. In our previous study, by utilizing the Illumina HiSeq and PacBio sequencing platforms combined with Single-Molecule Real-Time (SMRT) sequencing technology to screen for differentially expressed genes regulating the development of lpls common buckwheat mutants with equal-length stamens and pistils, we identified the FaesSEP4_3 gene. The expression of FaesSEP4_3 showed significant differences in the stamens and pistils of different floral types of common buckwheat during key developmental stages, suggesting its potential involvement in the morphological differentiation of floral organs in different floral types. Clarifying the role and function of the FaesSEP4_3 gene in regulating the morphological differentiation of floral organs in lpls common buckwheat plants will accumulate data for deciphering the floral development of lpls common buckwheat.

2. Materials and Methods

2.1. plant Materials

lpls common buckwheat was cultivated on the balcony of Yangtze University. During the full flowering stage and fruit setting stage, samples were collected from seven tissues of the lpls common buckwheat plants, including roots, stems, young leaves, tepals, stamens, pistils, and 6-day-old fruits (achenes). Additionally, flower buds from five different developmental stages were sampled during the inflorescence development and floral bud differentiation periods of lpls common buckwheat. Total RNA was extracted from the tissue samples using liquid nitrogen grinding according to the instructions of the EASYspin Plant RNA Rapid Extraction Kit. Furthermore, flower buds from the five developmental stages were fixed in FAA solution [V(formaldehyde):V(glacial acetic acid):V(70% ethanol) = 1:1:18], and the

developmental stages of the five flower buds were identified through paraffin sectioning.

2.2. Cloning of the FaesSEP4_3 Gene in Common Buckwheat

Specific primers (GSPFaesSEP4_3) were designed in the 5' untranslated region (5'-UTR) using Oligo 7.0 software, following the primer design guidelines provided in the 3'-Full RACE Core Set Ver.2.0 Kit (TaKaRa). Total RNA was extracted from the flower buds of lpls common buckwheat using the EASYspin Plant RNA Rapid Extraction Kit, and the integrity and concentration of the RNA were assessed. First-strand cDNA for 3'-RACE was synthesized from the common buckwheat flower bud RNA using the 3'-Full RACE Core Set Ver.2.0 Kit (TaKaRa). The full-length sequence of the FaesSEP4_3 gene in lpls common buckwheat was obtained via PCR amplification.

The PCR products were separated by 1% agarose gel electrophoresis. The target bands with the correct size and clear resolution were excised, and the target fragments were purified and recovered using a DNA recovery kit. The purified fragments were ligated into the pTOPO-TA vector, and the resulting recombinant plasmids were transformed into E. coli F-TOP10 competent cells (Kingory, Wuhan). Positive clones were screened on ampicillin (AMP) plates, and the selected monoclonal colonies were cultured for colony PCR verification. Clones with correct verification results were sent to Wuhan Kingory Biological Engineering Co., Ltd. for sequencing, and the full-length sequence of the FaesSEP4_3 homologous gene in common buckwheat was successfully cloned.

Primer name	Primer sequences(5′—3′)	Application
GSPFaesSEP4_3	AGAGACGAACCAAATTCTTTC	3'RACE
QFaesSEP4_3F	CCAGAGAAAGGAGGAAGAACTCG	qRT-PCR
QFaesSEP4_3F	GCAACCACTCCGTCGCATATC	
QFaesActinF	ACCTTGCTGGACGTGACCTTAC	Internalreference
QFaesActinR	CCATCAGGAAGCTCATAGTTC	

2.3. Phylogenetic Tree Construction of FaesSEP4_3 in Common Buckwheat

The amino acid sequence encoded by the open reading frame (ORF) of the FaesSEP4_3 homologous gene in common buckwheat was first subjected to a BlastP homology search in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Over 30 homologous FaesSEP4_3 proteins from various plant species were selected. A molecular phylogenetic tree was then constructed using the Neighbor-Joining (NJ) method in MEGA 12.0 software. Additionally, homologous proteins from four species Arabidopsis thaliana, Malus pumila, Phalaenopsis, and Cymbidium sinense were selected. The ClustalW program within BioEdit 7.0 software was used to analyze the domains of the FaesSEP4_3 transcription factor.

2.4. Analysis of Physicochemical Properties, Secondary Structure, and Tertiary Structure Prediction of FaesSEP4_3 in Common Buckwheat

The basic properties of the FaesSEP4_3 gene sequence in common buckwheat were analyzed using the ProtParam tool in the ExPASy database (http://web.expasy.org/protparam). Subsequently, the protein's secondary structure was analyzed using the SOPMA tool, and the tertiary structure of the FaesSEP4_3 protein was predicted using the SWISS-MODEL website.

2.5. Expression Pattern Analysis of the FaesSEP4_3 Gene

Using the RNA extracted from the seven different tissues and five flower buds at different developmental stages as templates, the first-strand cDNA was synthesized with the HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing). Forward and reverse primers, qFaesSEP4_3F and qFaesSEP4_3R, were designed at the 3' end of the FaesSEP4_3 gene. Real-time quantitative PCR (qRT-PCR) was performed using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing), with the common buckwheat Faesactin (HQ398855.1) gene as the internal reference. This was done to detect the expression specificity of the FaesSEP4_3 gene in the seven aforementioned tissues and the dynamic changes in its expression levels across the five different flower bud developmental stages. The relative gene expression levels were calculated using the 2-ΔΔCt method. Statistical analysis was performed using SPSS 27.0 software, and the Least Significant Difference (LSD) method was used to analyze the significance of differences in the expression levels of the FaesSEP4_3 gene in common buckwheat.

3. Results and Analysis

3.1. Cloning of the full-length cDNA of the FaesSEP4_3 gene from common buckwheat

The full-length cDNA of the FaesSEP4_3 gene was cloned from lpls common buckwheat using 3' RACE technology. Sequence analysis was performed using the "ORF Finder" program in the NCBI online database (https://www.ncbi.nlm.nih.gov/). The results indicated that the sequence of the FaesSEP4_3 gene is 916 bp in length, containing a 35 bp 5'-UTR, a 690 bp open reading frame (ORF), and a 191 bp 3'-UTR region. It encodes a total of 229 amino acids and one stop codon.

3.2. Protein sequence alignment and molecular phylogenetic analysis

The phylogenetic tree (Figure 1) grouped the FaesSEP4_3 transcription factor into SEP4 lineage of the SEP subfamily within the MADS-box transcription factor family. The FaesSEP4_3 gene belongs to the SEP4 clade of the MADS-box gene family and was therefore named FaesSEP4_3. Protein sequence alignment analysis (Figure 2) showed that the FaesSEP4_3 transcription factor contains a highly conserved

MADS-box domain composed of 62 amino acid residues (1-62) and a secondary conserved K-domain composed of 80 amino acids (94-173). Moreover, two conserved motif domains, SEP I and SEP II, are located at the C-terminal region of the FaesSEP4_3.

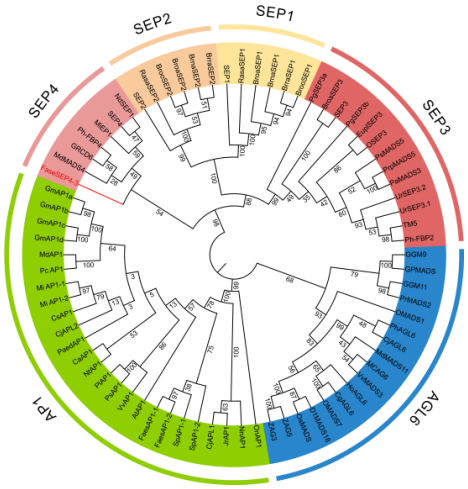


Fig 1. Phylogenetic analysis of FaesSEP4_3 with other proteins

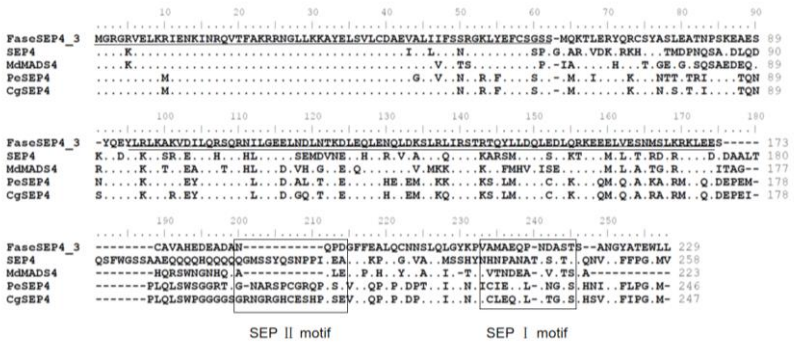


Fig 2. Alignment of putative amino acid sequences of FaesSEP4_3 with other homologous protein sequence

Single underlines indicate the MADS-box domain, double underlines represent the K-domain, and boxes denote the two conserved SEP I and SEP II motifs.

3.3. Analysis and prediction of physicochemical properties, secondary structure, and tertiary structure of the FaesSEP4_3 gene from common buckwheat

The predicted molecular formula of the FaesSEP4_3 protein is C1136H1840N328O369S9, with a relative molecular weight of 26.3 kD and a theoretical isoelectric point (pI) of 5.38, indicating that it is an acidic protein. The instability index is 49.91, suggesting that the protein is unstable. The aliphatic index is 84.41, and the grand average of hydropathicity (GRAVY) is -0.713, indicating that it is a hydrophilic protein.

The secondary structure analysis of FaesSEP4_3 showed that alpha-helices

accounted for the largest proportion at 51.09%, followed by random coils at 40.61%, and extended strands at 8.30%. Figure B shows the predicted tertiary structure of the FaesSEP4_3 protein.

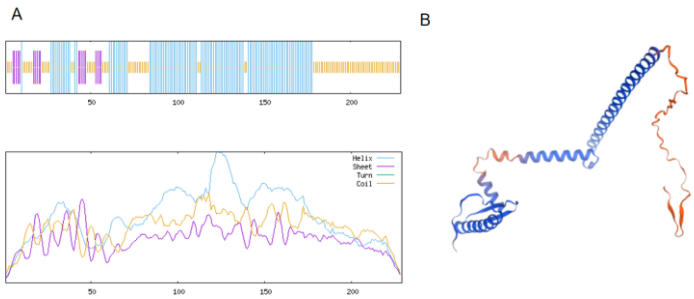


Fig 3. Secondary and tertiary structure prediction of FaesSEP4_3 protein Note

A: Secondary structure. B: tertiary structure

3.4. Analysis of the expression pattern of the FaesSEP4_3 gene in homostylous common buckwheat

qRT-PCR suggested that the FaesSEP4_3 gene is mainly expressed in roots, tepals, stamens, pistils, and fruits. Moreover, expression levels of FaesSEP4_3 in tepals, stamens, pistils, and fruits was obviously higher than that of the roots. Among floral organs, the expression level of FaesSEP4_3 is the highest in stamens, which is significantly higher than that in tepals and pistils ($p < 0.05$).

In addition, FaesSEP4_3 is obviously expressed throughout all stages of flower bud development. Obvious expression of FaesSEP4_3 was detected when the stamen and pistil primordia appear. FaesSEP4_3 expression continues to increase from microspore mother cell formation (S2) and reaches its peak on the day of anthesis during floral bud differentiation.

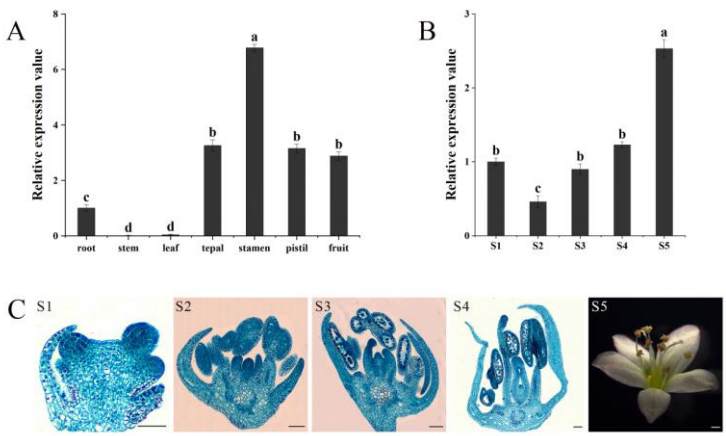


Fig 4. Expression of FaesSEP4_3 in lpls flower buckwheat

A. Differences in the expression levels of the FaesSEP4_3 gene across seven different tissues of lpls common buckwheat. The letters above the bars indicate the significance of differences among different tissues.

B. Dynamic changes in the expression levels of the FaesSEP4_3 gene during five different stages of floral bud differentiation in lpls common buckwheat.

C. Paraffin section images of lpls common buckwheat floral buds at four different stages prior to differentiation, and one image of the flower morphology on the day of anthesis. Scale bar: 100 μm.

S1. Stage of stamen primordia appearance; S2. Stage of microspore mother cell formation; S3. Stage of microspore tetrads; S4. Stage of uninucleate microspore formation; S5. Floral buds on the day of anthesis.

4. Discussion and Conclusion

In Arabidopsis thaliana, SEP4 works together with the other SEP genes (SEP1, SEP2, and SEP3) to regulate the development of sepals, petals, stamens, and carpels [9]. In Isatis indigotica, the SEP4-like gene IiSEP4 was obviously expressed in sepals, and its mRNA was mildly detected in leaves, inflorescences, flowers, stamens, and young silicles, indicating the important roles of IiSEP4 in floral development of Isatis indigotica [10]. In Cymbidium sinense, the SEP4-like gene CsSEP4 was expressed throughout all stages of flower bud development, and CsSEP4 was specifically highly expressed only in the gynostemium of floral organs; CsSEP4 works with other SEP-like genes to regulate floral organ development and flowering time [11-12]. In Prunus mume, the expression of the SEP4-like gene PmSEP4 was notably detected in the sepals and was involved in specifying flower meristem and sepal development [13]. In Tagetes erecta, the SEP4-like gene TeSEP4 was mainly expressed in the pappus and corollas, as well as regulates flowering transition and floral organ identity development [14]. In Gossypium hirsutum, the SEP4-like genes GhSEP4.1 and GhSEP4.2 were abundantly transcribed in the shoot apical meristem (SAM) and regulate flowering time [15]. In Dianthus chinensis, the SEP4-like genes DcaMADS6, DcaMADS7, and DcaMADS8 were expressed mainly in the sepals, and DcaMADS7 and DcaMADS8 were additionally expressed in petals [16]. In Brassica rapa, the SEP4-like genes (Bra026543, Bra025126, and Bra017376) were extremely highly expressed in the pistil and regulate pistil development [17]. In Prunus persica, the SEP4-like gene PaMADS2 was highly expressed during fruit development and ripening [18].

In our study, FaesSEP4_3 is mainly expressed in roots, tepals, stamens, pistils, and fruits. Moreover, expression level of FaesSEP4_3 in tepals, stamens, pistils, and fruits was obviously higher than that of the roots. In addition, FaesSEP4_3 is obviously expressed throughout all stages of flower bud development. These data suggests that FaesSEP4_3 gene may be involved in floral organ and fruit development of common buckwheat.

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