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Cloning and Functional Analysis of Flavonol Synthase Gene *ZjFLS* from Chinese Jujube (*Ziziphus jujuba* Mill.)

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Abstract

Flavonoids are an important type of bioactive substance contained in jujubes. Flavonol synthase (FLS) is a key enzyme for the synthesis of flavonoids such as flavonols and anthocyanins. To study the biological functions of *FLS* in jujubes, we cloned the *ZjFLS* gene; analyzed its physicochemical properties and evolutionary relationships; and then conducted an expression characteristic analysis, subcellular localization, prokaryotic expression and heterologous overexpression in *Arabidopsis thaliana*. The results showed that the length of *ZjFLS* is 951 bp, and it encodes 316 amino acids. A sequence analysis revealed that *ZjFLS* exhibited a high degree of conservation in evolution. The results of a qRT-PCR analysis indicated that the *ZjFLS* gene could be expressed in different tissues of jujube: the expression level was the highest in the leaves, followed by the flowers, and the lowest was in the fruits. Within these expression levels, it was higher in young leaves than in mature leaves and higher in the white-ripe-stage fruits than in the semi-red-stage fruits. Subcellular localization indicated that the *ZjFLS* gene was located in the nucleus, cytoplasmic matrix, and cytoplasmic membrane. Our research findings show that the ZjFLS protein can be induced and obtained in the prokaryotic expression system and successfully purified. It mainly exists in the form of inclusion bodies and has a relatively low content in the soluble supernatant. The total flavonoid content of *Arabidopsis thaliana* strains with a heterologous overexpression of the *ZjFLS* gene was significantly higher than that of the wild type, confirming that the *ZjFLS* gene can promote the biosynthesis of flavonoid substances.

Keywords: *Ziziphus jujuba* Mill.; *ZjFLS*; gene cloning; expression analysis; functional identification

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1. Introduction

Chinese jujube (*Zizihpus jujuba* Mill.) belongs to the *Ziziphus* genus of the Rhamnaceae family. It is the largest dry fruit native to China and an economically important forest tree species [1]. With a cultivation area of over 3.3 million acres and an annual output of over 8 million tons, it is the main economic source for over 20 million farmers. Therefore, the jujube industry plays a crucial role in the growth of the rural economy and the development of China's economy. The cultivation history of the jujube is long and its germplasm resources are rich. So far, more than 1000 varieties or superior types of jujube have been discovered and recorded [2,3]. Jujube fruit is honored as a traditional 'homology

of medicine and food'-type nutritious food and has become an indispensable ingredient on the dining tables of ordinary families. In the field of traditional Chinese medicine, it is often used as a 'an ingredient added to enhance the efficacy of a dose of medicine' due to the rich bioactive substances it contains [4], such as flavonoids [5], polysaccharides [6], vitamins [7], cyclic nucleotides [8], triterpene acids [9], and other functional nutrients [10]. Flavonol substances such as rutin and quercetin are important flavonoid components of jujubes. They have attracted much attention due to their biological functions, such as antioxidation, anti-inflammation, lipid-lowering, blood coagulation, hypoglycemic, and anti-cancer properties [11–13]. Flavonols are important substances involved in the normal growth and development of plants and various metabolic regulatory pathways, and they also play an important role under abiotic stress. However, at present, there are few reports on the regulation of flavonol synthesis in jujubes.

The flavonol metabolic pathway is an important branch of the flavonoid metabolic pathway, and flavonol synthase (FLS) is a key enzyme in the flavonol synthesis pathway and belongs to the 2-oxoglutarate-dependent dioxygenase (2-ODD) group [14]. *FLS* can convert dihydroflavonols into flavonols and is a key rate-limiting enzyme that determines the biosynthesis reaction of flavonol substances [15,16]. *FLS* has been widely studied in many plants. Firstly, the full length of *FLS* was cloned in petunias [17]. Subsequently, *FLS*s from various plants such as apple [18], banana [19], Tartary buckwheat [20], rhododendrons [21], lily [22], and grape hyacinth [23] were cloned and identified. Many studies have confirmed that *FLS*s are highly correlated with the accumulation of flavonols and other flavonoids. In grape hyacinths, *FLS*s are mainly expressed in the early stage of flower development; hetero-overexpression in tobacco significantly upregulates the expression of *NtFLS*, resulting in a significant increase in the total flavonoid content [24]. The expression of the three *FLS* genes in *Camellia sinensis* leads to the accumulation of flavonols [25]. The two genes, *FLS* and *DFR*, compete for the common substrate dihydroflavonol, which may lead to a decrease in anthocyanin content by regulating the expression of *FLS*. In crabapples, when *FLS* is overexpressed or *DFR* is silenced, the flavonol content in the fruits significantly increases [26]. The *FLS* and *DFR* genes of raspberries (*Rubus chingii* Hu) are highly expressed in stems, leaves, and flowers. However, the accumulation of flavonols in these organs is significantly higher than that of proanthocyanidins. Low concentrations of flavonols could significantly inhibit the activity of *DFR*. Studies have shown that *FLS* is in an advantageous position in competition with *DFR*, and the competition between these two genes regulates the metabolic flux distribution of flavonols and proanthocyanidins [27]. Generally speaking, the *FLS* of angiosperms seems to be more inclined towards dihydrokathol as the substrate, while *DFR* is more inclined towards dihydroquercetin and dihydromyricetin [28]. The expression of *FLS* can be induced by salicylic acid, abscisic acid, ultraviolet rays, low temperatures, ethylene glycol, etc. In grapes, exogenous salicylic acid may regulate the biosynthesis of flavonols in the fruit by activating *FLS* activity at specific developmental stages, and the accumulation of flavonols may be involved in the formation of salicylic-acid-mediated acquired resistance [29].

Flavonoids play a significant role in the growth, development, and stress resistance of jujubes. *FLS* is located at a key hub in the flavonoid metabolic pathway, which has been extensively documented to be closely associated with flavonoid biosynthesis. However, the function and regulatory mechanisms of *FLS* in jujube flavonoid synthesis remain unelucidated. Therefore, cloning and functional research on the *FLS* gene in jujubes has a very important theoretical basis and application value for improving functional nutritional quality and stress resistance. Through the transcriptomal analysis of jujube fruits at different developmental stages, an *FLS* gene was screened and its expression level during fruit development was positively correlated with the contents of flavonol

substances such as rutin and quercetin; hence, it was speculated that this gene may play a key role in the synthesis of flavonol substances. A bioinformatics analysis has become one of the core components of modern-life scientific research. Conducting a bioinformatics analysis on a gene not only allows for the quick location of its basic information but also helps in gaining deeper insights into gene functions or guiding experimental designs [30]. In this study, cloning and bioinformatics analyses of this gene were conducted and its function was preliminarily verified through an expression characteristic analysis, examining subcellular localization, prokaryotic expression, and heterologous transgenics; this could lay a theoretical foundation for the quality breeding of jujubes.

2. Materials and Methods

2.1. Plant Materials

The experiment was conducted at the Key Laboratory of Germplasm Improvement and Utilization in Pomology, Research Institute of Pomology, Shanxi Agricultural University of China, from 2022 to 2024. The test materials were collected from the National Jujube Germplasm Repository. The trees were 15 years old and the management conditions were consistent. The fruits of *Ziziphus jujuba* cv. Hupingzao in the white-ripe stage, semi-red stage and full-red stage, as well as the flowers, young leaves, and mature leaves, were selected as the experimental materials. *Arabidopsis thaliana* Col-0 and *Nicotiana tabacum* L. plants were individually cultivated in plant growth chambers under standardized conditions of a 14 h light and 10 h dark photoperiod, maintaining humidity levels between 55% and 65%. The growth temperature for plants was maintained at 22 ± 2 °C. *Nicotiana tabacum* L. plants aged 4 weeks were employed for the subcellular localization analysis.

2.2. ZjFLS Cloning and Sequence Analysis

The RNA of the semi-red-stage fruit of *Ziziphus jujuba* cv. Hupingzao was extracted using an RNA extraction kit (Rapid Universal Plant RNA Extraction Kit 3.0, Beijing Huayueyang Quick RNA Isolation Kit, Beijing, China), and the cDNA obtained by reverse transcription was used as the template. Amplification primers were designed using the CDS sequence of the *FLS* gene, and screened out by the transcriptome. The primer sequence is as follows: FLS—F: AGAACACGGGGGACGAGCTCATGGGTTGCTCCGGCATTCC and FLS—R: ACCATGGTGTCGACTCTAGAGGGGGTAATTGCATAGTGAGTAATATTG. The PCR reaction system was 40 μL: including 20 μL of 2 × Hieff Canace® Gold PCR Master Mix (Shanghai, China), 2 μL of forward primers, 2 μL of reverse primers, 1 μL of cDNA template, and 15 μL ddH_2O. The PCR reaction procedure was as follows: pre-denaturation at 94 °C for 3 min; denaturation at 98 °C for 10 s, annealing at 68 °C for 1 min, and extension at 72 °C for 1 min for 35 cycles; and extension at 72 °C for another 5 min. The amplified fragments were subjected to 1.0 % agarose gel electrophoresis, and then the target fragments were recovered and purified using a DNA gel recovery kit (DP209, Tiangen Biology, Beijing, China). The target fragment was ligated with the clonal vector PCAMBIA2300-GFP (Beijing, China) and then transformed into *Escherichia-coli*-competent cells DH5α (DL1001, Weidi Biology, Shanghai, China). After plate inoculation, culture, plasmid DNA extraction, and PCR identification, the single colonies that tested positive with the PCR were activated and sent to Shanghai Bioengineering Company of China for sequencing. The BLAST function of NCBI was used to search for protein sequences, the SMART tool 1.9.7 was used for the domain analysis, and DNAMAN 8.0 was used for multiple sequence alignment. The physicochemical properties were analyzed using the online software ProtParam (https://web.expasy.org/protparam/ (accessed on 9 March 2024)). Secondary structure prediction was performed using SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/secpred_sopma.pl (accessed on 9 March 2024)). The signal peptide analysis was conducted

with SignalP-6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/ (accessed on 9 March 2024)). The phosphorylation site analysis was carried out using NetPhos-3.1 software (https://services.healthtech.dtu.dk/services/NetPhos-3.1/ (accessed on 9 March 2024)). The phylogenetic tree visualization analysis was performed using CLC Sequence Viewer 8.0 with a bootstrap value of 1000 and all other parameters set as defaults.

2.3. qRT-PCR Expression Analysis of ZjFLS

The total RNA extraction and cDNA synthesis of samples such as fruits at different developmental stages and different tissues were carried out in accordance with the method in Section 2.2. Based on the cloned *ZjFLS* sequence, we designed and screened out specific primers using PrimerQuest Tool (http://sg.idtdna.com/Primerquest/Home/Index (accessed on 11 April 2024)), as follows: qZjFLS—F: GCATATTGAATGAGTGCTTGGG, qZjFLS—R: TCTGTGGCTGGAAAGTAACG. Taking the *Actin* gene of jujube as the internal reference, we designed the primers of the internal reference genes (*Actin*—F: AGCCTTCCTGCCAACGAGT, *Actin*—R: TTGCTTCTCACCCTTGATGC). The amplification reaction was carried out using an AB-7500 real-time quantitative PCR instrument, and fluorescence quantitative detection was performed using Kit Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (Shanghai, China). The reaction system was 20 μL, including 10.0 μL of the mix, 0.4 μL of forward and reverse primers with a final concentration of 0.2 μmol/L, 2.0 μL of cDNA template (200 ng/μL), and 7.2 μL ddH_2O. Three biological repetitions were carried out for this experiment. The $2^{-\triangle\triangle CT}$ method was applied to analyze the data in order to determine the gene relative expression level [31].

2.4. Subcellular Localization of ZjFLS

Using pCAMBIA2300-GFP as the skeletal vector, the linearized vector was prepared through the double enzyme digestion of SacI and XbaI. The specific steps were as follows. Firstly, we designed specific primers for PCR amplification (upstream primer: 5′-AGAACACGGGGGACGAGCTC-3′, underlined at the SacI site; downstream primer: 5′-ACCATGGTGTCGACTCTAGA-3′, underlined at the XbaI site). Secondly, using the *ZjFLS* clone plasmid as the template, the target fragment was amplified by high-fidelity DNA polymerase. Thirdly, the amplification products were linked to the linearized vector through a homologous recombination system and transformed into *Escherichia coli* DH5α-competent cells. Finally, through colony PCR screening and Sanger sequencing verification, the recombinant plasmid pZjFLS-GFP was successfully obtained.

The recombinant plasmid pZjFLS-GFP and the empty vector control pCAMBIA2300-GFP were transformed into Agrobacterium root-cancer GV3101 receptor cells using the thermal shock method, and then successfully transformed Agrobacterium was obtained after sequencing verification. Next, *Nicotiana tabacum* L. that had grown to the 4–6 true-leaf stage was selected, and a 1 mL sterile syringe was used to infiltrate and inject the Agrobacterium suspension, with OD 600 = 0.6 (containing 150 μmol/L acetyleugenone), onto the backs of the leaves. After inoculation, the plants were cultivated under conditions of 22 °C and 16 h of light/8 h of darkness for 48–72 h. The infected leaf tissues were taken to create temporary slides, and the GFP signals were observed using a Leica SP8 (Oberkochen, Germany) confocal laser microscope. DAPI (PI62247, Thermo Fisher Scientific, Waltham, MA, USA) nuclear staining was set up as a reference for nuclear localization.

2.5. Prokaryotic Expression Analysis of ZjFLS

Based on the sequence information of *ZjFLS* obtained by sequencing, we designed specific primers. The upstream primer was pET-ZjFLS-F: 5′-GGACAGCAAATGGGTCGCGGATCCATGGGTTGCTCCGGCATTC-3′ and the downstream primer was pET-ZjFLS-R: 5′-GTGGTGGTGGTGGTGCTCGAGGGGGGTAATTGCATAGTGAGTAATATTG-3′.

After obtaining the target gene fragment through PCR amplification, the target gene was directionally ligated to the pET-28a (+) expression vector and linearized by the double enzyme digestion of *BamH* I and *Xho* I using homologous recombination cloning technology. After verification by colony PCR and confirmation by sequencing, the recombinant expression vector pET—28a—ZjFLS was successfully constructed. The recombinant plasmid was transformed into competent *Escherichia coli* BL21(DE3) cells using the thermal shock method and then spread onto an LB solid medium containing 50 μg/mL kanamycin, which was inverted and cultured at 37 °C for 12–16 h. Single colonies were picked and inoculated into an LB liquid medium containing the corresponding antibiotics. After they had been shaken and cultured at 37 °C and 220 rpm until the logarithmic growth phase (OD 600 = 0.6–0.8), isopropyl-β-D-thiogalactoside (IPTG) was added until the final concentration was 0.5 mM for the induced expression. The induction culture was kept at 37 °C for 4 h. We centrifuged it at 4 °C and 5000× *g* for 10 min to collect the bacteria. The bacterial precipitates were resuspended with a pre-cooled Tris-NaCl buffer (20 mM Tris-HCl, 150 mM NaCl, and pH 8.0) and ultrasonically fragmented under ice-bath conditions. After centrifuging the crushed liquid at 12,000× *g* and 4 °C for 30 min, the supernatant (soluble protein) and the precipitate (inclusion body) were collected, respectively. The recombinant protein was purified by nickel ion affinity chromatography, the supernatant was loaded onto the pre-balanced HisTrap HP column (GE Healthcare, Chicago, IL, USA), and gradient elution was performed, successively using the binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, and pH 8.0) and the elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, and pH 8.0). The following samples were analyzed by 12% SDS-PAGE electrophoresis: pre-induction bacterial solution, post-induction bacterial cells, ultrasonic disruption supernatant, centrifugal precipitation, and purified protein samples. Then, the expression of the target protein was observed after Coomassie brilliant blue R-250 staining.

2.6. Acquisition of Arabidopsis thaliana Strains Overexpressed by ZjFLS

The constructed *ZjFLS*-pCAMBIA2300 recombinant plasmid was transformed into *Arabidopsis thaliana* by the Agrobacterium-mediated flower-soaking method, and the heterologous overexpressed positive strains were identified.

The harvested *Arabidopsis thaliana* seeds were added to sterile water to remove surface contaminants and disinfected with 70% ethanol and a 7% sodium hypochlorite solution (containing one drop of Twain) for 10 min and then spread evenly onto 1/2 MS culture plates (the screened antibiotic added was 30 μg/mL hygromycin) for germination to screen for resistant overexpressed strains. We observed after 8 to 15 days and selected the resistant seedlings that had grown true leaves and could take root for transplanting. We extracted DNA from the leaves of different transgenic seedlings and wild-type *Arabidopsis thaliana*, and conducted conventional PCR to amplify the target gene (the primers and procedures used were equivalent to those in Section 2.2) to confirm if heterologous overexpressed strains were obtained. The seeds of the positive seedlings were collected as individual plants. The T1 transgenic *Arabidopsis thaliana* underwent further resistance screening until the T3 generation homozygous strains were obtained.

2.7. qPCR Detection of ZjFLS Gene Expression Level and Determination of Total Flavonoid Content in Positive Arabidopsis thaliana Strains

We extracted total RNA from the leaves of wild-type *Arabidopsis thaliana* strains and some homozygous transgenic strains and reverse-transcribed them to obtain cDNA. Using the obtained cDNA as a template and the *Arabidopsis thaliana EF1α* gene as an internal reference, specific primers (F: CACCACTGGAGGTTTTGAGG; R: TGGAGTATTTGGGGGTGGT) were designed. The fluorescence quantitative primers for overexpressing the *Arabidopsis thaliana* gene *ZjFLS* were the same as those in Section 2.3. Each

biological sample was repeated three times. The qRT-PCR test was conducted in accordance with the instructions for the Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) kit, and the reaction system, reaction procedure, and calculation method of the relative expression level were the same as those in Section 2.3.

The determination of the total flavonoid content in *Arabidopsis thaliana* was carried out using the $NaNO_2$-$AI(NO_3)_3$-NaOH colorimetric method, referring to the method of Zhao et al. [32], and optimized and improved on this basis. The specific procedure is as follows. Extract the total flavonoid using 80% methanol to obtain the total flavonoid extract. Transfer 0.5 mL of the extract to a 10 mL volumetric flask, add 80% methanol to bring the volume to 5 mL, and then add 0.3 mL of 10% $NaNO_2$. Mix well and let stand for 5 min. After that, add 0.3 mL of 10% $AI(NO_3)_3$, mix well, and let stand for another 5 min. Then, add 2 mL of 8% NaOH, followed by dilution to the 10 mL mark with 80% methanol. Allow the mixture to stand for 15 min and measure the absorbance at 510 nm. Use the treatment without NaOH as the blank control. Draw the standard curve with rutin as the standard substance.

3. Results

3.1. Cloning and Bioinformatics Analyses of the ZjFLS Gene

We amplified the *FLS* gene using the cDNA of the semi-red-stage fruit of *Ziziphus jujuba* cv. Hupingzao as a template and the designed specific primers. The results of the electrophoresis determination combined with a sequencing analysis indicated that the cloned sequence was consistent with the expected target (Figure 1). The length of the cloned *FLS* is 951 bp, with predicted encoding of 316 amino acids; the protein molecular weight is 36.20 kD; and the theoretical isoelectric point (pI) is 5.77. A protein secondary structure analysis revealed that ZjFLS was composed of 33.54% α-helix, 16.46% β-fold, and 50.00% random curl. This protein has no signal peptide and its phosphorylation site is mainly serine.

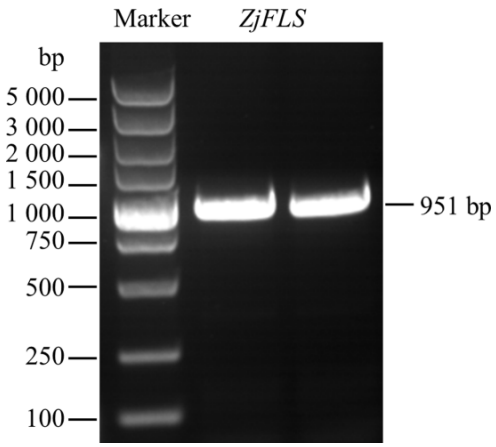

Figure 1. The electrophoresis of PCR amplification of *ZjFLS*; 2 kb plus a DNA marker were utilized for agarose gel electrophoresis.

3.2. Analysis of ZjFLS Sequence Characteristics

We searched different plant FLS sequences within the NCBI database. Eight species, *Pistacia vera*, *Eucalyptus grandis*, *Psidium guajava*, *Acer negundo*, *Cajanus cajan*, *Quercus robur*, *Carya illinoinensis*, and *Tripterygium wilfordii*, were screened out based on multiple restrictive conditions such as coverage, sequence consistency, and required length, and then the sequences of these species were compared with ZjFLS in multiple sequences (Figure 2). It can be seen that the amino acid sequence encoded by the *ZjFLS* gene is highly

similar to the FLS amino acid sequence of other species, indicating that the function of the ZjFLS protein in jujubes is relatively conformed.

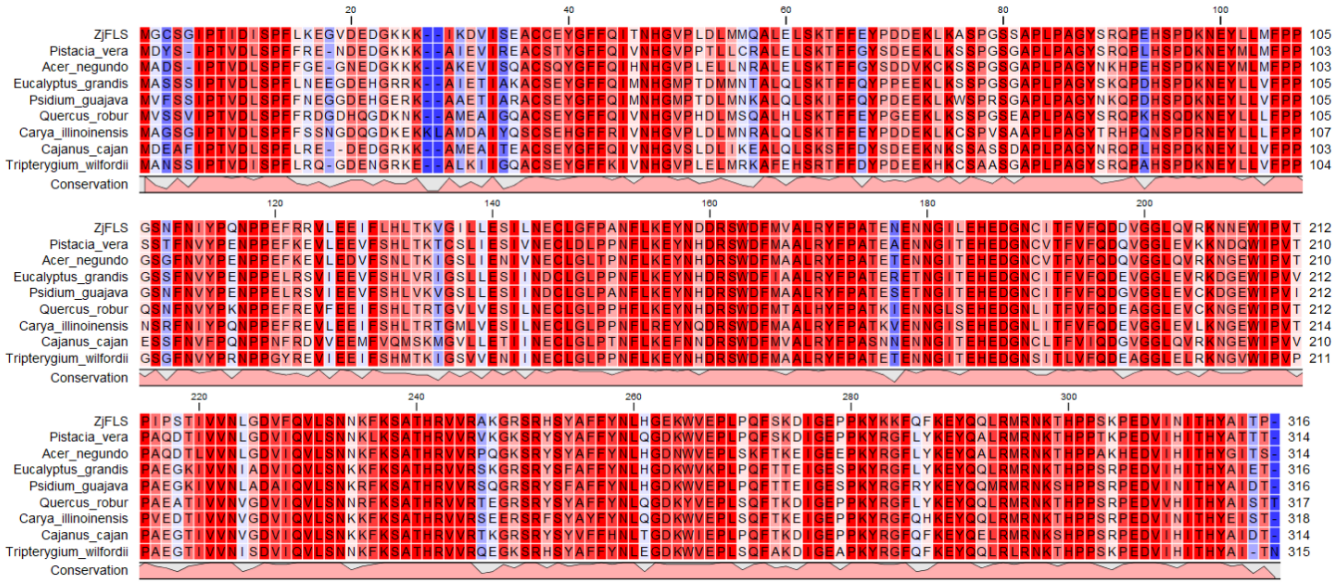

Figure 2. Alignments of protein sequences of ZjFLS and other FLSs.

Based on the protein sequence of ZjFLS, a BLAST comparison was conducted in NCBI, and we downloaded homologous genes of a total of eight species, including *Pistacia vera*, *Eucalyptus grandis*, *Psidium guajava*, *Acer negundo*, *Cajanus cajan*, *Quercus robur*, *Carya Illinoinensis*, and *Tripterygium wilfordii*. We conducted a phylogenetic analysis of these homologous genes and the *ZjFLS* gene and found that *ZjFLS* has a relatively close genetic relationship with *Pistacia vera* and *Acer negundo* (Figure 3). This may be because of a relatively recent common ancestor, less convergent evolution, or less hybridization interference.

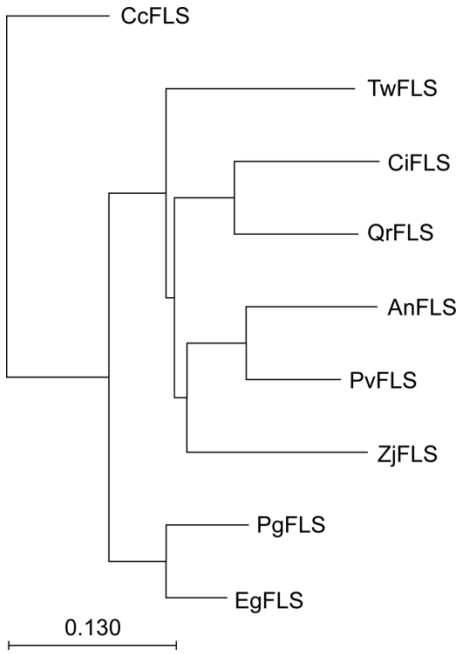

Figure 3. Phylogenetic analysis of ZjFLS with proteins from other species. Cc: *Cajanus cajan*; Tw: *Tripterygium wilfordii*; Ci: *Carya illinoinensis*; Qr: *Quercus robur*; An: *Acer negundo*; Pv: *Pistacia vera*; Zj: *Ziziphus jujuba*; Pg: *Psidium guajava*; Eg: *Eucalyptus grandis*.

3.3. Tissue and Organ Expression Characteristics of ZjFLS

The expression characteristics of *ZjFLS* in fruits at different developmental stages and different tissues of *Ziziphus jujuba* cv. Hupingzao were analyzed (Figure 4). The results showed that the *ZjFLS* gene could be expressed in fruits, leaves, and flowers, but the expression levels in different tissues greatly varied. For fruits at different developmental stages, the expression level of *ZjFLS* in the white-ripe stage was significantly higher than that in the half-red stage and full-red stage, while there was no significant difference between the half-red stage and full-red stage. For different tissues, the expression level of *ZjFLS* was the highest in leaves and was significantly higher in young leaves than in mature leaves; in mature leaves, it was significantly higher than in flowers. The expression level of *ZjFLS* in flowers was slightly higher than that in white-ripe fruits, but the difference was not significant. It can be found from this that the expression of *ZjFLS* has tissue specificity. The expression level is the highest in leaves, followed by flowers, and the lowest is in fruits.

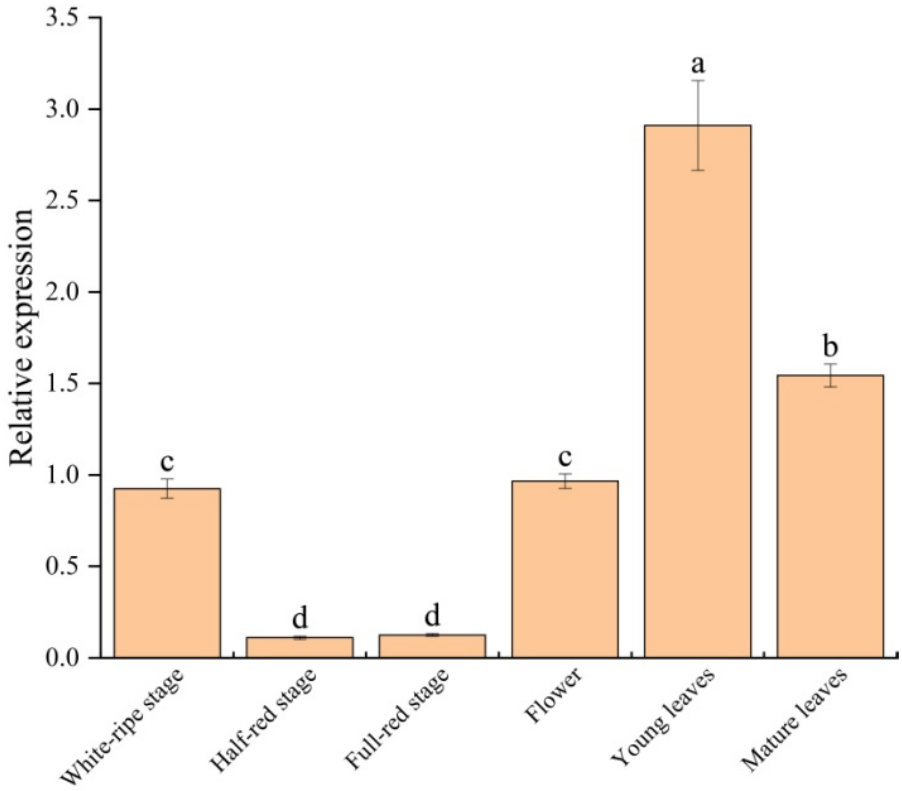

Figure 4. Expression analyses of *ZjFLS* gene in different developmental stages of the fruits and tissues of *Ziziphus jujuba* cv. Hupingzao. Different letters above the bars indicate significant differences at the level of $p < 0.05$.

3.4. Subcellular Localization of ZjFLS Protein

In order to understand the location of the ZjFLS protein in the cells, Agrobacterium containing the recombinant plasmid pZjFLS-GFP was injected into the leaves of *Nicotiana tabacum* L. and the empty plasmid pCAMBIA2300-GFP was used as the control. Then, the fluorescence position was observed by confocal microscopy. As can be seen from Figure 5, ZjFLS has no specific localization. In addition to being located in the cell nucleus, it can also be located in other parts of the cell, such as the cytoplasmic matrix and the cell membrane.

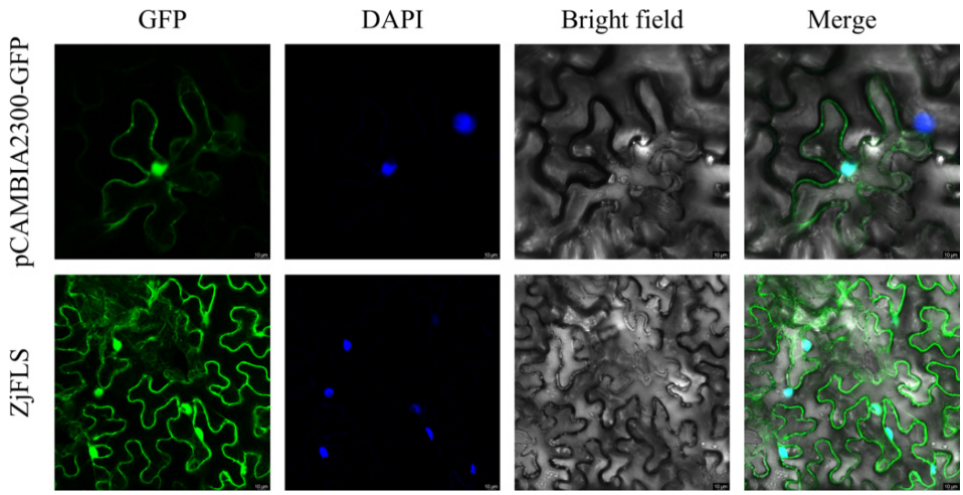

Figure 5. Subcellular localization analysis of ZjFLS in tobacco. The GFP channel denotes ultraviolet excitation, DIC corresponds with bright field, and Merge represents the overlay image. DAPI signifies the nuclear dye. The excitation wavelengths are 488 nm for the GFP channel and 358 nm for the DAPI channel. he scale bar in the image is 10 μm.

3.5. Prokaryotic Expression Analysis of ZjFLS

The protein expression analysis results based on SDS-PAGE indicated that the recombinant protein ZjFLS successfully achieved heterologous expression in the *Escherichia coli* BL21(DE3) system. The ZjFLS recombinant protein showed an obvious specific band four hours after being induced, and its apparent molecular weight was approximately 39 kDa (Figure 6), which was basically consistent with the theoretical molecular weight predicted based on amino acid sequences (39.46 kDa). The ZjFLS recombinant protein mainly exists in centrifugal precipitation, indicating that the protein is mainly expressed in the form of inclusion bodies. However, only trace amounts of ZjFLS were detected in the soluble supernatant. After purification by nickel column affinity chromatography, recombinant ZjFLS protein with good singularity could be obtained.

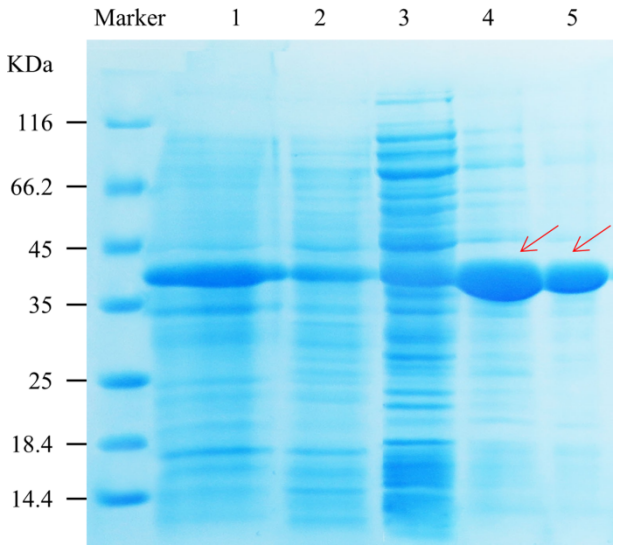

Figure 6. Expression and purification of ZjFLS in prokaryotes. Lane M denotes the protein marker. Lane 1 exhibits proteins from the lysed sample's pellet, Lane 2 displays proteins from the lysed sample's supernatant, Lane 3 shows proteins from the flow-through buffer, Lane 4 illustrates proteins from the wash buffer, and Lane 5 demonstrates proteins eluted from the purification bands. Red arrows point to the purified ZjFLS protein band.

3.6. Identification of Arabidopsis thaliana Strains with Heterologous Overexpression of the ZjFLS Gene

The *ZjFLS* gene was transformed into *Arabidopsis thaliana* by the floral dip method, and the transgenic strains were detected by PCR. The agarose gel electrophoresis results indicated that bands of the same size as the target gene fragment were obtained (Figure 7), suggesting that the *ZjFLS* gene was successfully integrated into the *Arabidopsis thaliana* genome and, therefore, we had obtained positive overexpressed *Arabidopsis thaliana* strains.

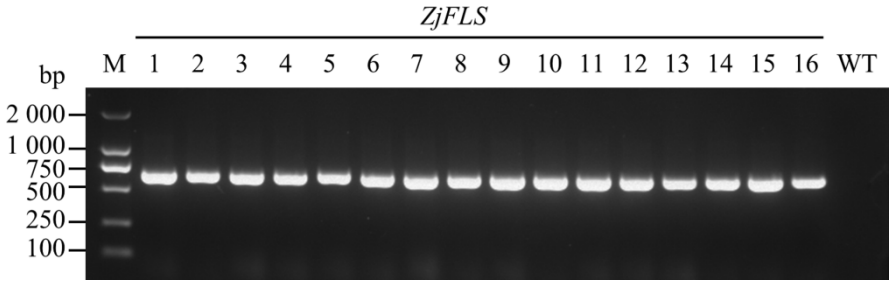

Figure 7. Identification of *ZjFLS* heterologously overexpressed *Arabidopsis thaliana.* M represents the marker and Lines 1 to 16 represent transgenic *ZjFLS* gene-positive lines. WT: wild type.

3.7. Analysis of the Expression Level of ZjFLS and Total Flavonoid Content in Positive Arabidopsis thaliana Strains

By analyzing the relative expression levels and total flavonoid content levels of wild-type and positive *Arabidopsis thaliana* strains (Figure 8), it could be seen that the total flavonoid contents of the nine positive *Arabidopsis thaliana* strains were all higher than that of the wild type. Among them, the total flavonoid content of strain 8 was the highest (2.510 mg/g FW), 3.67 times that of the wild type, and strain 9 was the lowest (1.333 mg/g FW), 1.95 times that of the wild type. The expression level of the *ZjFLS* gene in different strains was significantly positively correlated with the total flavonoid content, while the expression level in the wild type was 0. In conclusion, the *ZjFLS* gene plays an important role in the synthesis of flavonoids in Chinese jujube.

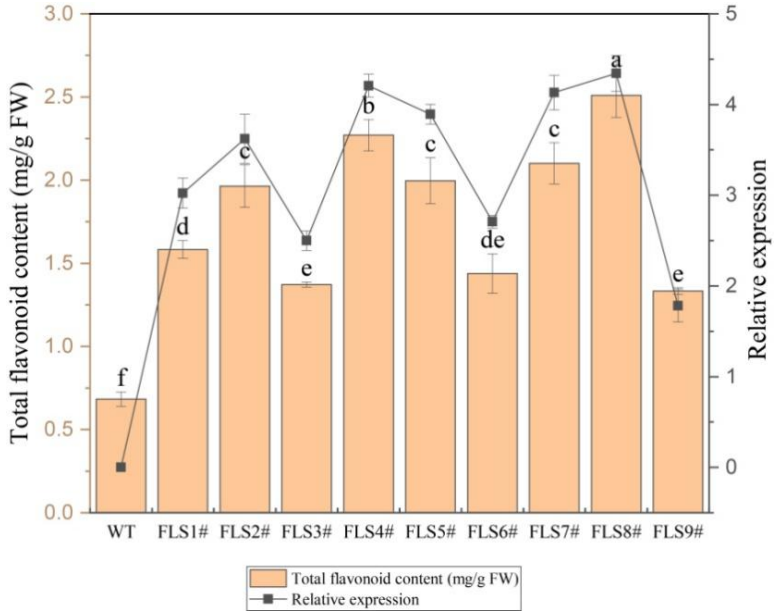

Figure 8. Expression levels of *ZjFLS* gene and total flavonoid content in *Arabidopsis thaliana* with overexpressed *ZjFLS*. Different letters above the bar indicate significant differences at the level of $p < 0.05$.

4. Discussion

Chinese jujube (*Ziziphus jujuba* Mill.) is an economically important forest tree species with strong stress resistance. Its fruit is rich in nutrients and is an important 'homology of medicine and food' fruit. Flavonoids are an important type of secondary metabolite contained in jujubes. *FLS*, as a key gene influencing the formation of flavonoids, has been isolated from a variety of plants. Combined with the results of previous studies, we screened out an *FLS* gene from the transcriptome data that might play a key role in the biosynthesis of flavonoids. To further study its biological functions, we cloned an *FLS* homologous gene from jujube fruits and named it *ZjFLS*. A multiple sequence alignment analysis showed that the amino acids encoded by this gene had high homology with the FLS amino acids of several other plants, indicating that the *ZjFLS* gene was relatively conserved among different species, which was the same as in previous research results. The results of the evolutionary tree showed that ZjFLS has a relatively high homology and similarity with five other species, including *Pistacia vera*, *Acer negundo*, *Quercus robur*, *Carya illinoinensis*, and *Tripterygium wilfordii*. The subcellular localization results indicated that the ZjFLS protein had signals in the nucleus, cytoplasmic matrix, and cytoplasmic membrane, which was consistent with the research results of Li Wei et al. on wheat FLS [33]. These research results all indicate that the *ZjFLS* gene plays a role in multiple parts of the cell.

Gene expression related to flavonoid compound biosynthesis is affected by plant species, tissues, and environmental conditions, and there are different regulatory mechanisms at work in plants. The expression level of the *FLS* gene varies in different developmental stages of plants, different tissues, and different environments. Through the expression analysis results of *ZjFLS* in this study, it can be known that its expression exhibits certain tissue specificity. Overall, the order of expression levels of *ZjFLS* in different tissues was leaves > flowers > fruits, and the expression level in young leaves was significantly higher than that in mature leaves. During the fruit development process, the expression level of *ZjFLS* demonstrated a dynamic change trend, with the highest expression level during the white-ripe stage. Many scholars have conducted extensive studies on the expression characteristics of the *FLS* gene in different species. For example, the highest expression level of *FLS* in *Yulania denudata* was found in flowers, and *Yulania liliiflora* had the highest expression level in young leaves [34]. This might be caused by the genetic differences among different plants. Among the three different flower colors of *Meconopsis* species, *FLS* was expressed in different tissues and the expression levels were significantly different [35]. The *SsFLS2* gene was expressed in the roots, stems, and leaves of *Solanum sisymbriifolium* Lam., and the relative expression levels in the roots and leaves were significantly higher than those in the stems [36]. The *FLS* gene was expressed in all tested tissues of blueberries and it could be seen that its expression level was particularly high in the petals and young leaves, while the expression level was relatively low in the fruits [37]. The above research results are basically consistent with those of this study. Therefore, the expression of *FLS* in different species shows tissue specificity.

Gene overexpression is widely used in the functional study of plant genes as the metabolic changes it causes can be manifested in the form of phenotypic changes or physiological data changes [38]. In apples, the overexpression of the proton pump gene *MdMa12* increased the malic acid content in both tomato and apple callus tissues [39]. The overexpression of the *MdmiR156n* gene promoted the accumulation of flavonoids and the clearance of reactive oxygen species (ROS) in transgenic apple calluses under drought conditions and *Arabidopsis thaliana* [40]. In grapes, after the overexpression of the *VvbZIP36* gene, the quercetin content increased due to the activation of the VvFLS promoter [41]. The overexpression of the *CsPALs* gene in citrus not only increases the phenolic substance content, but also causes changes in the contents of various sugars and increases most amino

acid contents [42]. When the *CHS* gene was overexpressed, the contents of flavonoids such as glycyrrhizin and isoglycyrrhizin, as well as total flavonoid in the hairy roots of transgenic licorice, were significantly higher than those in the wild-type hairy roots [43]. The results in this study indicate that the overexpression of *ZjFLS* can promote an increase in the total flavonoid content in *Arabidopsis thaliana*, which is consistent with the research results in safflower and ferns [44,45]. In sweet potato, silencing the *IbFLS* gene suppresses the expression of *IbFLS* and upregulates the expression of *DFR*, *ANS*, and *UFGT*, which results in a significant increase in total anthocyanin content and a decrease in total flavonol content in the leaves of transgenic plants. Thus, *IbFLS* participates in the flavonol biosynthesis pathway and is a potential candidate gene for color modification in sweet potato [46]. The overexpression of a certain gene can also affect the expression changes of other related enzyme genes in the synthetic pathway. In this study, the overexpression of the *ZjFLS* gene increased the total flavonoid content in *Arabidopsis thaliana*. But which flavonoids increased? This doubt creates the need for further studies in the future. Transcription factors are a class of gene regulatory molecules that bind to DNA and either activate or suppress the transcription of specific genes. In *Astraglus membranaceus*, *AmMYB35* promotes the expression of *FLS* by binding to its promoter. Under drought stress, flavonol accumulation is induced through the *AmMYB35–AmFLS* module, thereby enhancing drought tolerance by reducing ROS accumulation [47]. Therefore, in the future, we should conduct in-depth research into the interaction mechanisms of different genes and analyze the transcriptional regulation of related genes from the perspective of transcription factors. The aim is to provide a solid theoretical basis for the in-depth revelation of the molecular mechanisms of flavonoid substance synthesis in Chinese jujube.

5. Conclusions

The *ZjFLS* gene of jujubes was successfully cloned, with a length of 951 bp and encoding 316 amino acids. We conducted a bioinformatics analysis of the amino acid sequence of ZjFLS and the results indicated that the sequence was conserved. The *ZjFLS* gene is expressed in the leaves, flowers, and fruits and is tissue-specific. The expression level was the highest in the leaves, followed by the flowers, and was relatively low in the fruits. Subcellular localization revealed that the ZjFLS protein was located in the nucleus, cytoplasmic matrix, and cell membrane. The recombinant protein ZjFLS was successfully heterologously expressed in *Escherichia coli*, and its purified protein was obtained, providing a basis for further verification of its catalytic activity. The *ZjFLS* gene was overexpressed in *Arabidopsis thaliana*. Compared with the wild type, the total flavonoid content of the positive transformed strains significantly increased, confirming that the *ZjFLS* gene can play an important role in the flavonoid accumulation of jujubes. Next, we will conduct research into the influence of the *ZjFLS* gene on the synthesis of flavonoid monomers and its transcriptional regulatory mechanism.

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