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Identification and Functional Characterization of the *RcFAH12* Promoter from Castor Bean in *Arabidopsis thaliana*

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Abstract: Castor (*Ricinus communis* L.) seed oil is the commercial source of ricinoleate, a valuable raw material used in many industries. Oleoyl-12-hydroxylase (*Rc*FAH12) is a key enzyme in the biosynthesis of ricinoleate, accumulating nearly 90% of the triacylglycerol in castor seeds. Little is known about the transcriptional regulation of *RcFAH12*. We used rapid amplification of cDNA 5′ ends (5′RACE) to locate the transcription start site (TSS) of *RcFAH12*, and the sequence of a 2605 bp region, −2506~+99, surrounding the TSS was cloned. We then investigated these regions to promote β-glucuronidase (GUS) expression in transgenic *Arabidopsis* by the progressive 5′ and 3′ deletions strategies. The GUS staining showed that the GUS accumulation varied in tissues under the control of different deleted fragments of *RcFAH12*. In addition, the GUS expression driven by the *RcFAH12* promoter markedly accumulated in transgenic seeds, which indicated that *RcFAH12* might play an important role in the biosynthesis of ricinoleic acid. This study will lay a potential foundation for developing a tissue-specific promoter in oil-seed crops.

Keywords: castor bean; promoter; oleoyl-12-hydroxylase; RACE; multiple transcriptional start sites

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1. Introduction

Castor (*Ricinus communis* L.) is a diploid (x = 10, 2*n* = 20) oil plant in the Euphorbiaceae family. Its seeds contain a special hydroxylated fatty acid ricinoleate, also known as ricinoleic acid (18:1-OH; 12-hydroxy-9-cis-octadecenoic acid) which accounts for approximately 81.44–90.25% of the total fatty acids [1]. Castor oil has a wide range of applications and can be applied to produce coatings, biopolymers, paints, adhesives, cosmetics, lubricants, hydraulic oils, inks, linoleum, sebacic acid, and undecane carbon which can produce plasticizers and nylon [2]. It also can be used for nanoparticle preparation [3], treatment of blepharitis [4], and stimulate labor [5].

Since the widespread function of ricinoleic acid in industrial and medical applications, it is critical to reveal the biosynthesis of ricinoleic acid. In the early stages of castor seed

development, little ricinoleate is detected [6–8]. Most of the ricinoleate is synthesized in the mid-development of a castor seed, progressively increasing and reaching about 90% of the total fatty acid. It was reported that oleate 12-hydroxylase (*Rc*FAH12), a fatty acid modification enzyme, can convert the Sn-2-oleoyl group of phosphatidylcholine (PC) into the Sn-2-ricinoleyl group and act in a critical role in the biosynthesis of ricinoleate and high-level accumulation [9]. Compared with other enzymes involved in the biosynthesis of ricinoleate which displays a bell-shaped expression pattern, the expression level of *RcFAH12* gradually increases with the development of castor seeds [7]. Since the ricinoleate mainly accumulated in the seeds, it is not surprising that *RcFAH12* has almost undetectable transcription levels or lower expression in other tissues compared to the seeds [10,11]. However, until now, the promoter activity of *RcFAH12* and the possible seed-specific cis-acting elements have not been precisely investigated. In this study, we cloned and analyzed the *RcFAH12* 5′ upstream region and tested whether these regions possessed the promoter activity in transgenic *Arabidopsis* by the progressive 5′ and 3′ deletions strategies. This study will increase understanding of the mechanism of the high expression of *RcFAH12* in seeds and reveal the reason for the differential expression of *RcFAH12* among the tissues.

2. Results

2.1. Sequence Analysis of RcFAH12

The *RcFAH12* 5′ and 3′ terminal sequences were obtained using RACE, and the full-length cDNA of *RcFAH12* (deposited at NCBI: Genebank no. MK913424.1, https://www.ncbi.nlm.nih.gov/nuccore/MK913424.1, accessed on 15 December 2019.) in castor bean was obtained. By comparison with the genome sequence (NW_002994581.1, KC972615.1), the primary transcript form of *RcFAH12* (deposited at NCBI: Genebank no. MK913425.1, 7dacc9448127bdd3, accessed on 15 December 2019) (Figure 1) consists of two exons and one intron with a 2438 base-pairs (bp) 5′ untranslated region (5′UTR), 183 bp 3′ untranslated region (3′UTR), and 1164 bp open reading frame (ORF) encoding 387 amino acids corresponding to a 44.44 kDa protein. The exons are 127 and 1373 bp, separated by one intron of 2285 bp. The sequence at the 5′ end of the intron is GU, and the sequence at the 3′ end is AG. The polyadenylation signal (AATAAA) in the *RcFAH12* cDNA was present at 101 bp before the poly-A tail. The TSS was located 153 bp upstream of the translation start codon in mature mRNA.

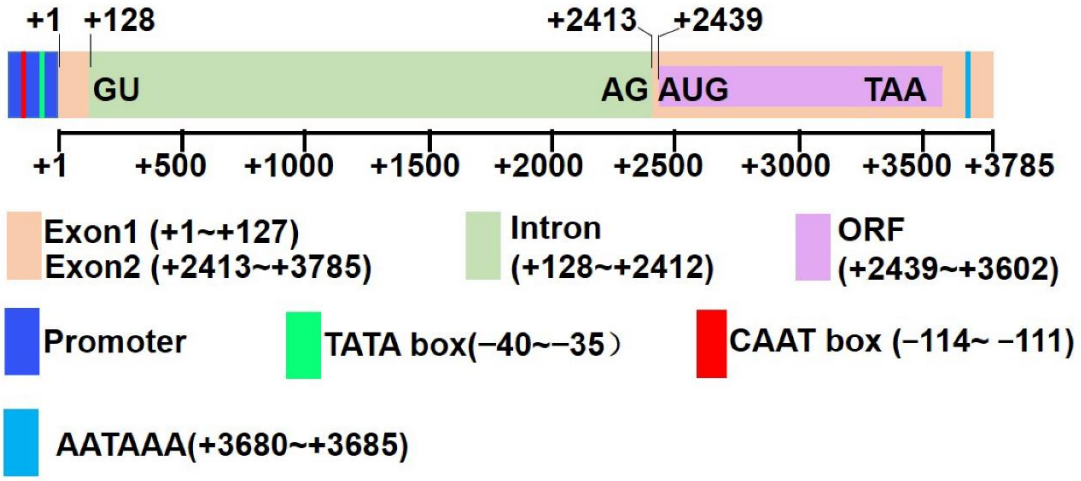


Figure 1. The structure of *RcFAH12*. The primary transcript form of *RcFAH12* consists of two exons and one intron with a 2438 bp 5′ untranslated region (5′UTR), 183 bp 3′ untranslated region (3′UTR), and 1164 bp ORF. The exons are 127 and 1373 bp, separated by one intron of 2285 bp. The sequence at the 5′ end of the intron is GU and the sequence at the 3′ end is AG. The polyadenylation signal (AATAAA) in the *RcFAH12* cDNA was present at 101 bp before the poly-A tail.

2.2. Sequence Analysis of the RcFAH12 Promoter

The TSS of most eukaryotic genes is not far upstream of the initiation codon. Researchers typically select approximately 2 kb of sequence upstream of the initiation codon to use as the promoter [12–14]. However, *RcFAH12* has an intron (2285 bp) upstream of the initiation codon, and the TSS is at 127 bp upstream of this intron. In addition, the core promoter encompasses ~50 bp upstream and ~50 bp downstream of the TSS [15,16]. For these reasons, the 2605 bp sequence contains 2506 bp upstream and 99 bp downstream of the TSS was selected for analysis.

The TSSs of *RcFAH12* are predicted by TSSPlant analysis [17]. Using TSSPlant analysis, there exists two TATA promoters and four TATA-less promoters. The TSSs of the TATA promoter are 2503 bp and 915 bp, and the TATA-less promoter TSSs are 2148 bp, 1792 bp, 1216 bp, and 315 bp, respectively, from 5′ to 3′ of the sequence 1~2605 bp (Supplementary Figure S1). The TSS is located at 2503 bp and is the fourth base upstream of the TSS determined by RACE above.

PlantCARE analysis was carried out to predict cis-acting elements in the *RcFAH12* promoter (Supplementary Figure S1). According to in silico analysis, 25 TATA-box and 30 CAAT-box exist in the plus strand of the *RcFAH12* promoter region (−2506~+99). The most likely locations of the authentic TATA-box and CAAT-box were selected at the −30 and the −111 positions based on the TSS and common regular patterns, respectively. In addition, the *RcFAH12* promoter also contains the cis-acting element involved in the abscisic acid responsiveness (ABRE), the cis-acting regulatory element essential for anaerobic (ARE) activity, the element for maximal elicitor-mediated activation (2 copies) (AT-rich sequence), the part of a conserved DNA module involved in light responsiveness (Box 4, GATA-motif, G-Box, GT1-motif, TCT-motif), the cis-acting regulatory element involved in methyl jasmonate (MeJA)-responsiveness (CGTCA, TGACG-motif), the cis-acting element involved in low-temperature responsiveness (LTR), the cis-acting regulatory element involved in zein metabolism regulation (O2-site), and the cis-acting element involved in salicylic acid responsiveness (TCA-element). It shows that the promoter may be affected by hormones such as abscisic acid, methyl jasmonate, salicylic acid, and environmental factors such as low temperature and anaerobic.

2.3. Deletion Analysis of the Promoter

The direct genetic transformation of castor bean is the main bottleneck for revealing the genes and their promoter functions although a meristem-based protocol has been established with low transformation efficiency (0.04%) [18]. In addition, the genetic transformation of the castor bean is restricted by its long-life cycle, poor repeatability, and high chimeras, which seriously hinders the molecular elucidation of the castor gene function. Therefore, *Arabidopsis* becomes an ideal alternative for functional studies of genes and promoters [13,19]. To locate the seed-specific intervals and possible cis-acting elements, 3′- and 5′-deleted promoter fragments were cloned due to the existence of multiple TSSs. We constructed plasmids containing deletion fragments of the *RcFAH12* promoter. To measure the transcriptional activity of these promoter sequences, we inserted them into the upstream region of the pCAMBIA1303 vector lacking the CaMV35S promoter elements. The plasmids were introduced into *Arabidopsis* by Agrobacterium-mediated plant transformation. To evaluate the activities of the *RcFAH12* promoter derivatives, different tissues of the transgenic *Arabidopsis* plants were used for histochemical staining to monitor the expression of the GUS gene (Figure 2). The histochemistry showed that the CaMV35S promoter strongly promoted the GUS reporter gene in all tissues. No GUS staining was observed in the untransformed wild-type *Arabidopsis*. *FP1611* drives the highest expression of GUS in rosette leaves, followed by *FP2605*, and the rosette leaf veins had very little GUS expression driven by *FP502*; no GUS gene expression was observed from the other deleted promoters. Except for *FP1611*, which could not drive GUS expression in anthers, all other deleted promoters could drive GUS expression in the early stages of anther developmental rather than in the later stages. *FP677* and *FP584* drive anther-specific expression of GUS

genes. *FP1611* can drive the expression of GUS in the calyx, petals, filaments, and stigma, but not in anthers; *FP2605* can promote the expression of the GUS gene in the calyx, stigma, and early stages of anthers development. *FP494* can drive GUS gene expression in the calyx and early stages of anthers development, but the expression levels are lower. The shortest *FP256* can drive GUS gene expression in *Arabidopsis* embryos, whereas the longer *FP677* cannot display GUS expression. *FP2605*, *FP1611*, *FP256*, *FP502*, and *FP613* could drive GUS gene expression in *Arabidopsis* embryos, and the expression levels were higher.

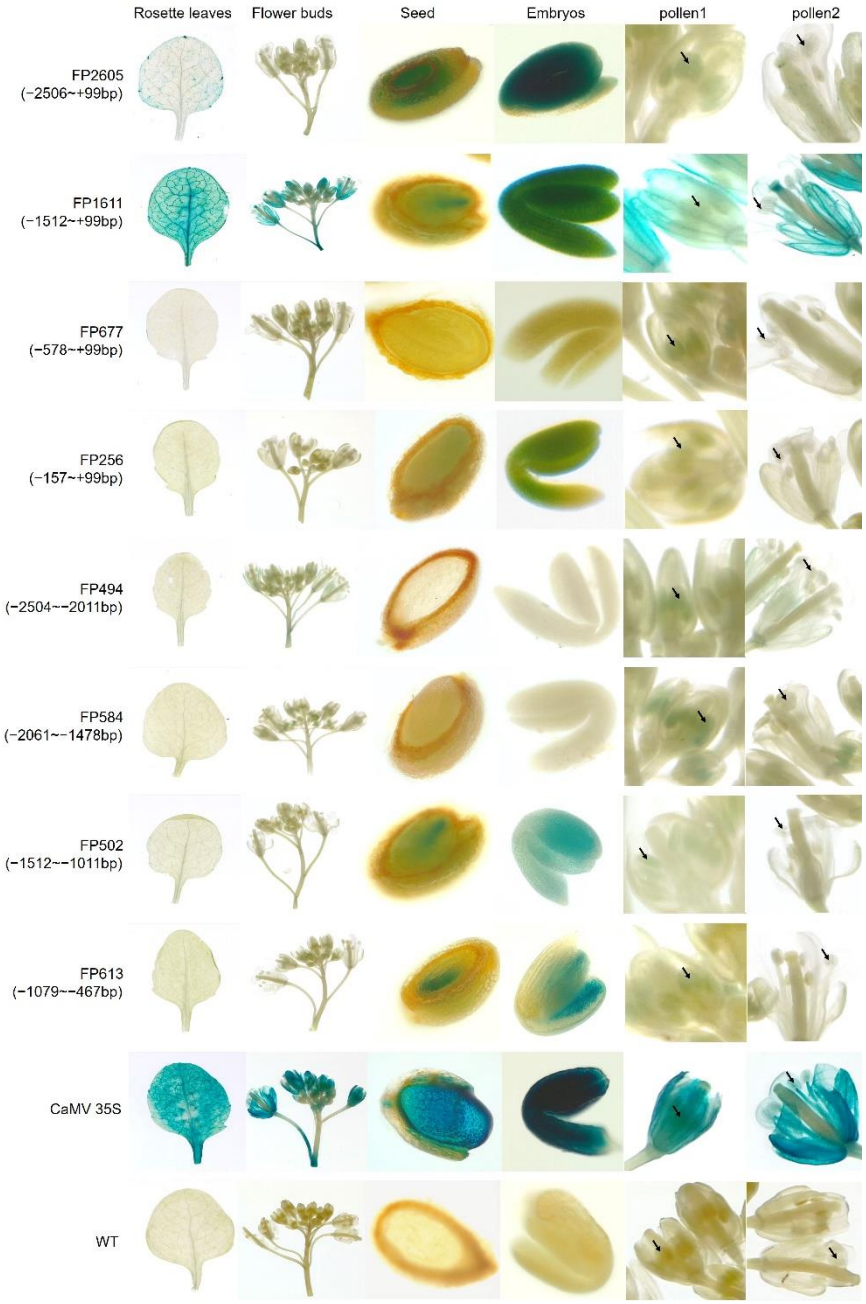


Figure 2. The GUS expression analysis in the transgenic plant containing different deletion fragments of the *RcFAH12* promoter. GUS staining results of different promoter fragments driving GUS gene expression in rosette leaves, flower buds, seeds, and embryos. CaMV 35S promoter as a positive control, wild type *Arabidopsis thaliana* without GUS gene as a negative control. Pollen1 and Pollen2 are enlarged images of early flowers and late flowers flower buds, respectively. (Arrows indicate the location of one of the anthers). CaMV35S strongly promoted the GUS reporter gene in all tissues. No

GUS staining was observed in the untransformed wild-type *Arabidopsis*. *FP1611* drives the highest expression of GUS in rosette leaves, followed by *FP2605*, and the rosette leaf veins had very little GUS expression driven by *FP502*; no GUS gene expression was observed from the other deleted promoters. Except for *FP1611*, which cannot be expressed in anthers, all other deletion promoters drive GUS expression in early anthers but not in later anthers. *FP677* and *FP584* drive anther-specific expression of GUS genes. *FP2605*, *FP1611*, *FP256*, *FP502*, and *FP613* drive GUS gene expression in *Arabidopsis* embryos, and the expression levels were higher.

3. Discussion

3.1. RcFAH12 Has Multiple TSSs

Gene expression is a highly complex process that underlies the fate and function of different cells and tissues. Regulation of this process consists of multiple horizontal molecular events [20–22]. The central event in regulating eukaryotic gene expression is the initiation of transcription by recruiting RNA polymerase II to the core promoter region [23]. In this study, the full-length cDNA of *RcFAH12* was obtained by RACE, and the TSS of *RcFAH12* was determined.

TSSPlant analysis showed that there were two TATA promoters and four TATA-less promoters in *FP2605*. In *FP494, FP584, FP502*, *FP613,* and *FP256,* each fragment contains a predicted TSS and each fragment independently drives GUS gene expression (Figure 2). In eukaryotic genes, transcription initiation often occurs at multiple TSSs, resulting in what is commonly known as promoter clusters [24]. Gene expression is controlled by multiple regulatory mechanisms, such as tissue-specific transcriptional activation, alternative splicing, and mRNA editing [25–27], and the selection of TSSs in response to environmental changes [28]. TSS selection influences transcript stability and translation as well as protein sequence [29]. Genes with higher expression levels tend to have a broader distribution of TSSs [30]. Therefore, multiple TSSs in the *RcFAH12* promoter may be involved in developmental regulation and response to environmental changes in the regulation of gene expression. This important finding reshaped the view on transcription initiation, showing that there is a higher complexity to this process than previously anticipated.

3.2. RcFAH12 Expression Is Subject to Complex Transcriptional Regulation

The mRNA level of *RcFAH12* showed a 268-fold difference in signal intensity between primary seed and leaf [31]. *RcFAH12* was expressed at negligible levels in leaves and young seeds, at a high level during cellular endosperm development, and with continuous sharp increases during the remaining stages. These findings indicate that the *RcFAH12* is transcriptionally expressed at similarly high rates throughout the cellular endosperm development and, at the same time, the *RcFAH12* transcripts are probably stable in the cellular endosperm, resulting in their high degree of accumulation [32].

To determine which sequence regions within *RcFAH12* promoter are required for seed-specific expression, we generated different deleted fragments of promoter. Surprisingly, *FP2605* and *FP1611* can drive strong expression of GUS in embryos whereas no GUS staining was observed in embryos under the control of *FP677*. However, *FP265* can drive the GUS expression in embryos, which indicates that some unknown sequence between −578~−158 might negatively affect seed-specific cis-elements. Lai et al. [33] reported that the AT-rich region in the promoter can negatively or positively affect gene expression depending on the position of the AT-rich region. We find that the AT-rich region accounts for 95% at the 5′ end of *FP677* (−549~−474 and −454~−364) whereas it accounts for 95% at the 3′ end of *FP613* (−549~−474), which indicates that the position of the AT-rich region might play a negative or positive role in the regulation of GUS expression driven by *FP677* or *FP613*, respectively. In addition, these results also indicated that the expression level would be different with the selection of TSS. Since the expression level of GUS driven by *FP1611, FP502,* and *FP613* highly accumulated in the transgenic seed compared with that controlled by *FP677*, this suggests that seed-specific cis-elements might be located in the sequence of −1512~−577. We found that the two cis-elements (G-BOX and ABRE)

involved in the regulation of seed expression [34,35] exist in *FP256*. Unsurprisingly, the GUS accumulation controlled by *FP256* can be found in seeds.

After deletion of −2506~−1513, *FP1611* cannot drive GUS gene expression in anther, while *FP494* and *FP584* have shown anther-specific expression. In addition, *FP584* drives more GUS gene expression in anther, indicating that −2506~−1513 contains anther-specific elements. It may be TGACG-motif/CGGTCA-motif. There is one TGACG-motif(+)/CGTCA-motif(−) on *FP494*, and two TGACG-motif(−)/CGTCA-motif(+) on *FP584*. Both TGACG-motif and CGTCA-motif are cis-acting regulatory elements involved in MeJA-responsiveness. After further deletion of −1512~−579, *FP677* showed anther-specific expression of GUS, indicating that there are seed-specific cis-elements in this sequence, and the deletion of these elements affects the expression of GUS gene in seeds.

Surprisingly, except *FP1611*, which can't drive GUS gene expression in *Arabidopsis* anthers, all other detected promoter fragments can drive GUS gene expression in the early stage of *Arabidopsis* anther development, but they are silent in the later stage. Among them, *FP677*, *FP494*, and *FP584* showed anther-specific expression of GUS gene. This result was different from the early research on the castor bean genome. Lipid analysis of the castor bean indicated that pollen and male developing flowers did not contain 18:1-OH. RNA-Seq transcriptomic analysis revealed a high expression of *RcFAH12* in developing seeds, but no *RcFAH12* was found in the male flower (Supplementary Figure S2a) [8]. However, the latest RNA-seq showed that a small expression of *RcFAH12* was detected in castor pollen (Supplementary Figure S2b) [11]. To find out the reason for this difference, we used quantitative real-time PCR to detect the expression of *RcFAH12* in the early male flower (i.e., before bud opening) and the late male flower (i.e., just after bud opening). The results showed that the expression of *RcFAH12* in the early male flower and the late male flower was strong and weak in the anthers, respectively (data not shown). Therefore, this difference may be caused by different sampling periods of male flowers in the early study. Each promoter deletion fragment has at least one TSS, and the GUS staining results show that different promoter deletion fragments can drive the expression of the GUS gene in *Arabidopsis* thaliana, indicating that this result may be related to the selected TSS [36,37]. The *RcFAH12* promoter deletions could drive GUS gene expression in anthers except for *FP1611*. The transcriptional regulation of this promoter also requires the participation of introns upstream of the translation initiation site [38,39]. In addition, some elements were associated with hormones, light, and stress tolerance. In summary, *RcFAH12* expression is subject to complex transcriptional regulation.

3.3. RcFAH12 Promoter Can Be Used for Genetic Engineering

The practical application of plant genetic engineering requires the efficient and stable expression of heterologous genes and production of target proteins in specific organs and tissues of transgenic plants [19]. The choices of promoters are very critical factors in determining the expression level and stability of heterologous genes. Constitutive promoters such as CaMV35S were extensively used to drive expression of heterologous genes. However, they can cause additional metabolic burden or toxic effects, ultimately leading to morphological and physiological dysfunction [40,41]. Compared with constitutive promoters, the tissue-specific and induced promoters can fine-tune gene expression at different developmental stages and abiotic stresses. In fact, improved promoters are always in demand because there is no 'perfect' promoter for a variety of cultural situations [42]. Although several seed-specific promoters of various oil crops are identified [12,43–46], the promoter that precisely regulates ricinoleic acid has not yet been reported. In our study, based on the promoter deletion strategies, we discovered that the GUS accumulation varied in tissues under the control of different deleted fragments of the *RcFAH12* promoter. In addition, the GUS expression driven by the *RcFAH12* promoter markedly accumulated in transgenic seeds, which indicated that *RcFAH12* might play an important role in the biosynthesis of ricinoleic acid. This study will lay a potential foundation for developing tissue-specific promoter in oil-seed crops.

4. Materials and Methods

4.1. Plant Material

The castor bean (genotype Tongbi 5) used in this study is a commercial crop with high oil content widely grown in Northeast China [47]. Fresh castor seeds and leaves were harvested from plants grown on the experimental farm of the Inner Mongolia Minzu University (43°36′ N, 122°22′ E) in the temperate continental monsoon climate region of China. Leaves were harvested at about 5 cm diameter and fruits were harvested 50 days after pollination (DAP). The samples were immediately frozen in liquid nitrogen and stored at −80 °C.

Wild-type and transgenic *Arabidopsis thaliana* ecotype Columbia plants were cultured in MS medium in a climate incubator and then transferred to pots with sterile soil for further growth at 22 °C at 16 h light/8 h dark cycles.

4.2. Rapid Amplification of cDNA Ends (RACE)

Since the current castor bean genome is only a draft, RACE technology was carried out to exactly clone the 5′ end of *RcFAH12* mRNA, which can determine the TSS of this gene.

4.2.1. Intermediate Fragment Cloning of *RcFAH12* cDNA

Total RNA was extracted from 50 DAP castor beans using TRIquick reagent (Solarbio, Beijing, China) with high salt buffer liquid [48]. Using the reported sequence of *RcFAH12* (U22378.1, https://www.ncbi.nlm.nih.gov/nuccore/U22378.1/, accessed on 14 July 1995 and XM_002528081.1, accessed on 6 August 2009. https://www.ncbi.nlm.nih.gov/nuccore/XM_002528081.1/), we amplified the intermediate fragment of cDNA by RT-PCR with the gene-specific primers C1 and C2 (Supplementary Table S1). PCR reaction procedure was as follows: an initial denaturation at 94 °C for 2 min, 30 cycles at 94 °C for 45 s, 59 °C for 45 s, 72 °C for 60 s, and a final extension step of 72 °C for 2 min. The PCR products were purified using a DNA Extraction Kit (Solarbio, Beijing, China), subcloned into the pMD® 18-T Vector (Takara, Beijing, China), and transformed into *E. coli* DH5α competent cells. Positive clones were identified by digestion with *Eco*R I/*Hind* III and sequencing.

4.2.2. Cloning of the 5′ and 3′ Terminal Sequences

The 5′ and 3′ terminal sequences were obtained using nested PCR with the 5′-Full RACE kit (Takara, Dalian, China) and the 3′-Full RACE Core Set (Takara, Dalian, China) with PrimerScript™ RTase (Takara, Dalian, China) according to the manufacturer's instructions.

For 5′ RACE, ligated RNA was obtained from 10 μL 50 DAP castor seed, which was dephosphorylated, decapped, and connected to the 5′ RACE Adaptor according to the manufacturer's instructions. The reverse transcription reaction solution was prepared according to the manufacturer's instructions and incubated at 30 °C for 10 min, 42 °C for 1 h, and 70 °C for 15 min to obtain cDNA with 5′ ends containing the adaptor. The obtained cDNA was used as a template for Outer PCR, and the primers were FWO and 5′ RACE Outer primer. The PCR reactions were performed at 94 °C for 2 min, 20 cycles at 94 °C, 45 s, 63 °C, 45 s, 72 °C, 1 min, and a final extension step of 72 °C, 7 min. The 1 μL Outer PCR product was used as template, FWI and 5′ RACE Inner primer as primers, and the PCR reaction was performed at 94 °C for 4 min, 30 cycles at 94 °C, 45 s, 60 °C, 45 s, 72 °C, 1 min, and a final extension step of 72 °C, 7 min. For 3′ RACE, 3 μL total RNA was mixed with 1 μL 3′ RACE Adaptor, denatured at 70 °C for 10 min, immediately bathed in ice water and then configured with reverse transcription reaction solution according to the instructions, and bathed in water at 42 °C for 60 min and 70 °C for 15 min. A cDNA containing a 3′ RACE Adaptor at the 3′ end was obtained. The 1 μL Outer PCR was performed using the obtained cDNA as template, and the primers were FSO and 3′ RACE Outer primer. The PCR reactions was performed at 94 °C, 2 min, 30 cycles at 94 °C, 45 s, 50 °C, 45 s, 72 °C, 1 min, and a final extension step of 72 °C, 2 min. The 1 μL Outer PCR product was used as template, FSI and 3′ RACE Inner primer as primers, and the PCR reaction was as follows: 94 °C, 2 min, 30 cycles at 94 °C, 45 s, 60 °C, 45 s, 72 °C, 1 min, and a final extension

step of 72 °C, 2 min. The sequence of primers used in RACE is shown in Supplementary Table S1. The PCR products were ligated to the pMD®18-T vector and transformed into *E. coli* DH5α, respectively. Positive clones were identified by digestion with *EcoR* I/*Hind* III and sequencing.

4.3. Cloning of the RcFAH12 Promoter and Sequence Analyses

After assembly with Contig Express Project software (Invitrogen, Carlsbad, CA, USA), the *RcFAH12* structure was obtained by comparison with the castor genome sequence (NW_002994581) and the 5′-UTR intron sequence of the *RcFAH12* (KC972615.1) [49]. Castor genomic DNA was isolated from the young leaves of Tongbi 5 plants using a Plant Genomic DNA Extraction Kit (Solarbio, Beijing, China). Based on the above results, the predicted *RcFAH12* promoter (*FP2605*, −2506~+99 bp) sequence was cloned by PCR with primer FAH12PF/FAH12PR (Supplementary Table S1) from castor genomic DNA and connected to the pMD®18-T vector. Reactions were performed with Takara *LATaq* DNA Polymerase (Takara, Beijing, China) under the following PCR conditions: initial denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 45 s, 56 °C for 45 s, 72 °C for 3 min, and a final extension step of 72 °C for 5 min. Positive clones were identified by digestion with *Pst* I/*Bgl* II and sequencing. The plasmid was named pMD18-*FP2605*. Since the *RcFAH12* TSS determined by RACE is downstream of the 5′ end of the submitted sequence (U22378.1 andXM_002528081.1), the sequence of the predicted *RcFAH12* promoter (−2506~+99 bp) was analyzed with TSSPlant (http://www.softberry.com/berry.phtml?topic=tssplant&group=programs&subgroup=promoter accessed on 6 August 2022) [17] for putative TSSs, and the promoter sequences were analyzed in detail by using the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ accessed on 6 August 2022) [50]).

4.4. Deletion Analysis of Cloned RcFAH12 Promoter Fragment

To design the primers, the In-Fusion® HD Cloning Kit (Takara, Beijing, China) user manual was referred to (Supplementary Table S1). The plasmid pMD18-*FP2605* was used as a template for amplification, and the corresponding FPF/FPR primer pairs were used to amplify the promoter deletion fragments. Cycling parameters were as follows: 30 cycles of 98 °C for 10 s, 60 °C for 3 min, and a final extension step at 4 °C. *FP2605* (−2506~+99 bp) was considered the longest promoter, while *FP1611* (−1512~+99 bp), *FP677* (−578~+99 bp), and *FP256* (−157~+99 bp) were 5′ truncated fragments (Figure 3). *FP494* (−2504~−2011 bp) was the 3′ truncated fragment, while *FP584* (−2061~−1478 bp), *FP502* (−1512~−1011 bp), and *FP613* (−1079~−467 bp) were truncated at both ends (Figure 3).

4.5. Construction of a Series of Promoters::GUS Vectors and Transformation of Arabidopsis

The PCR products of promoter deletions were purified using a DNA Extraction Kit (Solarbio, Beijing, China), subcloned into the pCAMBIA1303 that was previously digested with the *Pst* I/*Nco* I, and transformed into *E. coli* DH5α. PCR identified positive clones with primers 1303F/303R (Supplementary Table S1) and we sequenced the positive clones.

All the recombinant plasmids were transformed into *Agrobacterium tumefaciens* GV3101 strain using the freeze-thaw method. The *Arabidopsis thaliana* ecotype Columbia was transformed by the standard flower-dip protocol [51] using GV3101. The positively transformed plants were selected with 50 mg/L hygromycin to obtain T1 generation. Five T1 generation transgenic lines per final expression vector were first selected with 50 mg/L hygromycin and then confirmed by PCR amplification with qGUSF1/qGUSR1 (Supplementary Table S1) as primers.

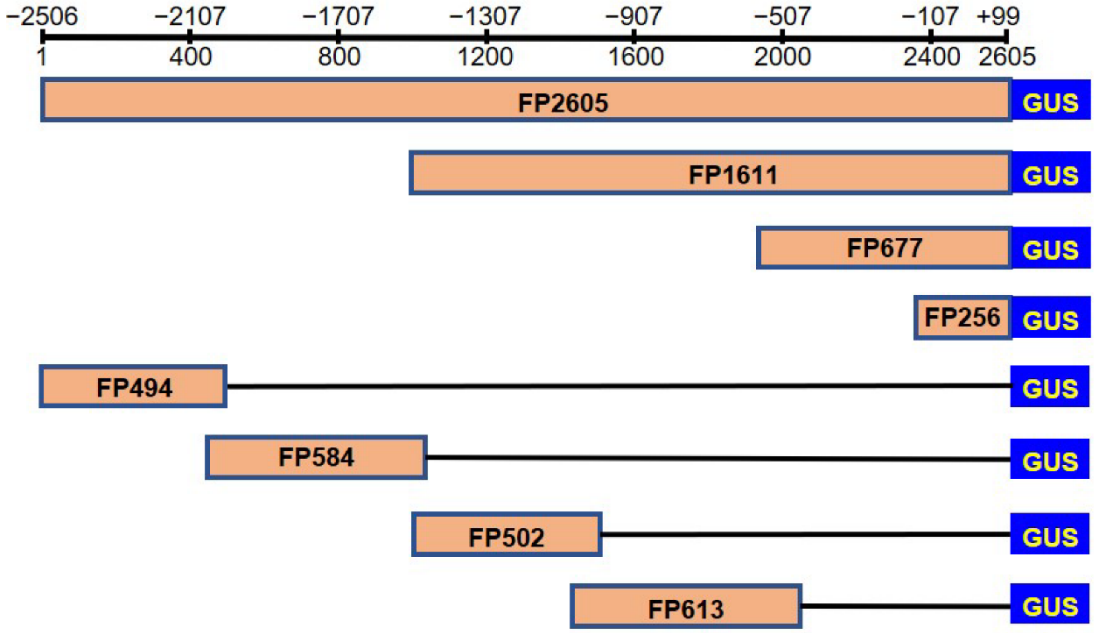


Figure 3. Construction of expression vectors. The sequences of *FP2605* (−2506~+99 bp), *FP1611* (−1512~+99 bp), *FP677* (−578~+99 bp), *FP256* (−157~+99 bp), *FP494* (−2504~−2011 bp), *FP584* (−2061~−1478 bp), *FP502* (−1512~−1011 bp), and *FP613* (−1079~−467 bp) were ligated to pCambia1303 to obtain pT2605, pT1611, pT677, pT256, pT494, pT584, pT502, and pT613.

4.6. GUS Histochemical Staining

The rosette leaves, flower buds, and siliques of T3 homozygous transgenic lines were collected for GUS staining [52] with modifications, and three biological replicates were carried out. The tissues were washed three times with 100 mM sodium phosphate buffer (pH 7.0) and then immersed in a GUS substrate solution consisting of 2 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-Gluc), 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 1 mM potassium ferricyanide, and 0.001% (v/v) Triton X-100. The siliques were processed by CM1950 (Leica, Germany) to expose the seeds and embryos to the mature green stage. The treated tissues were incubated at 37 °C overnight, followed by the removal of chlorophyll in 70% ethanol solutions. The GUS staining tissues were then examined by DVM6 (Leica, Wetzlar, Germany).

5. Conclusions

The present work determined the full-length sequence and transcription initiation site of *RcFAH12* from the castor bean. According to the TSS of *RcFAH12* and the genomic sequence, a promoter fragment (−2506~+99) from *RcFAH12* was cloned. Many cis-elements were found in the promoter fragment. Deletion analysis of the promoter in transgenic *Arabidopsis* showed that the different deletion fragments of *RcFAH12* promoters had relative seed-specific or anther-specific expression and multiple TSSs. These promoter fragments have the potential to be used in future genetic engineering.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10010002/s1, Figure S1: The cis-acting elements in *RcFAH12* promoter region. The positions and sequences of different cis-elements are marked with different colored backgrounds and their names. The blue font in the sequence shows the TATA box, the red font shows the CAAT box, and the green font is the overlapping area of the two. Red +1 is the RACE-determined TSS of *RcFAH12* in 50DAP seeds. Blue and purple +1 are TATA and TATA less TSS predicted by TSSPlant, respectively.; Figure S2: Heatmap of *RcFAH12* expression level (fragments per kilobase of transcript per million fragments sequenced, FPKM) in different tissues of castor bean.

This heatmap was drawn by TBtools software [53]; Table S1: Primer design with *RcFAH12* Promoter deletion mutant.

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