Plant Biotechnol Rep (2016) 10:369–381
DOI 10.1007/s11816-016-0407-6

Online ISSN 1863-5474
Print ISSN 1863-5466

CrossMark

ORIGINAL ARTICLE

The promoter of fatty acid desaturase on chromosome C5 in *Brassica napus* drives high-level expression in seeds

Fang Liu[1,2] · Guiluan Wang[3] · Ruiyang Liu[1,2] · Chunyun Guan[1,2]

Received: 30 December 2015 / Accepted: 13 September 2016 / Published online: 20 September 2016
© Korean Society for Plant Biotechnology and Springer Japan 2016

Abstract The gene fatty acid desaturase 2 (*FAD2*) exists in multiple copies in the *Brassica napus* genome and encodes an enzyme that catalyzes the conversion of oleic acid to linoleic acid. In the present study, we characterized the regulatory region controlling the expression of an *FAD2* gene located on chromosome C5 of *Brassica napus* and named it *BnFAD2-C5*. A long intron was found within the 5′-untranslated region (5′-UTR) of the *BnFAD2-C5* gene. This intron, compared with an intron-less control, conferred up to a sixfold increase in green fluorescent protein (GFP) expression in transgenic *Arabidopsis*, thus suggesting that it makes function through intron-mediated enhancement. The sequence containing the promoter and intron was identified to promote high levels of gene expression in genital organs, particularly in seeds, using qRT-PCR and transgenic *Arabidopsis*. We identified the different promoter regions responsible for the tissue-specific gene expression through a deletion analysis of the *BnFAD2-C5* promoter and a β-glucuronidase and GFP reporter system. The results showed that the −1020 to −319 bp region primarily controls *BnFAD2-C5* gene expression in the root, whereas the −1020 to −581 bp region controls expression in the stem, the −581 to −319 bp region controls expression in the leaf, and the −1257 to −1020 bp region probably controls expression in the floral parts. The −319 to −1 bp region is also important, conferring high-level transcription in the seeds. The transcription of *BnFAD2-C5* could be induced by salicylic acid and jasmonic acid, and the relative response elements were identified in the −1257 to −1020 bp region and −319 to −1 bp region, respectively.

Keywords *Brassica napus* · *Arabidopsis thaliana* · *BnFAD2-C5* · Intron-mediated enhancement

✉ Chunyun Guan
guancy2011@126.com

[1] College of Agronomy, Hunan Agricultural University, Changsha 410128, People's Republic of China

[2] The Oil Crops Research Institute/National Oil Crops Improvement Center, Changsha 410128, People's Republic of China

[3] State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian 271018, Shandong, People's Republic of China

Introduction

Plant lipids are altered to improve oil quantity and quality for using in food, bioenergy, and industrial bioproducts (Suh et al. 2015). Canola oils containing high oleic acid and low linoleic acid have attracted researchers' attention, owing to their attributes associated with oleic acid, including high oxidation stability, extended shelf life, and limited unpleasant odor (Maher et al. 2007). Some genes involved in the biosynthesis and accumulation of oleic acid have been target genes in studies seeking to improve the quality of rapeseed oils; however, little is known about the regulatory mechanisms of these genes. Studies on the regulation of these genes would provide a theoretical foundation for improving the quality of vegetable oils.

The oleic acid desaturase 2 (*FAD2*) gene encodes an enzyme that catalyzes the conversion of oleic acid to linoleic acid (Baud and Lepiniec 2010). Higher plants generally contain one or more *FAD2* gene copies, such as *Arabidopsis*, which has one copy of *FAD2* (Beisson et al. 2003); cotton, which has 3 copies (Pirtle et al. 2001);

sesame, which has one copy (Jin et al. 2001); soybeans, which have 2 copies (Bilyeu et al. 2003); flax, which has 2 copies (Chen et al. 2015); and *Brassica napus*, which has 4 copies (Lee et al. 2013). Different *FAD2* gene copies have different expression patterns. For example, in normal-type sunflower, the *FAD2-1* gene is highly expressed, especially in developing embryos, but the *FAD2-2* and *FAD2-3* genes are weakly expressed (Martínez-Rivas et al. 2001). In sesame, the *FAD2* gene is expressed with a seed-specific pattern (Kim et al. 2006).

Yang et al. (2011) have detected two major quantitative trait loci (QTLs) for oleic acid content in the linkage groups (LGs) of chromosomes A5 and C5, which explain 60–70 % of the variance in oleic acid content and are located in the *FAD2* gene. Moreover, cloning and aligning the coding sequences of the *FAD2* genes in high- and low- oleic acid rapeseeds indicate that the high oleic acid content might be explained by the expression of the *FAD2* gene. The expression of *FAD2* gene in high oleic acid rapeseed is lower than that in the standard rapeseed (Guan et al. 2012), and the oleic acid content can be significantly improved by knocking down *FAD2* expression using RNAi in *Brassica napus* and *Brassica campestris* (Jung et al. 2011; Peng et al. 2010). All the above findings suggest that the functions of the *FAD2* genes depend on their ability to be expressed efficiently. To improve oleic acid content in rapeseed, studying the expression and regulation patterns of both the *FAD2* genes is necessary. Xiao et al. (2014) have studied the transcription regulation of the *FAD2* gene on chromosome A5, which has a constitutive expression pattern and is regulated by positive and negative *cis*-elements. To explain the regulatory functions of both *FAD2* genes in fatty acid synthesis in *Brassica napus*, a study of the *FAD2* gene on chromosome C5 should be conducted to determine how *FAD2* is expressed and what causes it to be expressed, which would allow plants with the desired oil characteristics to be engineered.

Introns not only maintain the specific structure and function of chromosomes but also determine a gene's expression level and its spatial and temporal expression (Deyholos and Sieburth 2000). Introns are induced by internal and external signals and regulate spatial and temporal gene expression during seed development (Bolle et al. 1996; Kim et al. 2006; Xiao et al. 2014). The first intron found to stimulate gene expression was in an animal system, and the observation was extended to plants when Callis et al. (1987) demonstrated that the first intron of Adh1 in maize increases the expression of several genes. Introns with an enhancement function are usually located in the 5′-untranslated region (UTR) (Kim et al. 2006). Intron-mediated enhancement (IME) in dicots is usually in the range of approximately 2- to 10-fold and is much lower than that in monocots, which can reach more than 100-fold (Clancy and Hannah 2002). Typically, introns that are located nearer to the 5′end of a gene have a greater enhancement of expression than those at the 3′end (Parra et al. 2011). The 5′UTR of the *FAD2* gene on chromosome A5 in *Brassica napus* contains an intron that is responsible for IME (Xiao et al. 2014). Given the homology between chromosomes A5 and C5, the question arises as to whether the *BnFAD2-C5* intron might also be involved in IME and whether it can induce *BnFAD2-C5* gene expression spatially and temporally.

Internal and external signals are involved in the regulation of spatially and temporally expressed genes during plant growth. Abscisic acid (ABA) appears to play a key role in plant maturation and desiccation tolerance (Finkelstein et al. 2002). Moreover, the germination of seedlings is sensitive to ABA (Bäumlein et al. 1994). In addition, the repeated copies of ABREs can confer ABA responsiveness to minimal promoters, whereas a single copy of ABRE is not responsive to ABA (Narusaka et al. 2003). The plant hormones salicylic acid (SA) and jasmonic acid (JA) play key roles in the regulation of the defense-signaling network, which is activated in response to invaders (Pieterse et al. 2012). In general, pathogens with a biotrophic lifestyle are more sensitive to SA-induced defenses, whereas necrotrophic pathogens and herbivorous insects are resistant, owing to JA-mediated defenses (Glazebrook 2005; Howe and Jander 2008).

Here, we isolated the full-length *BnFAD2-C5* gene from the 5′UTR to the 3′UTR, including a long intron within the 5′UTR. The qRT-PCR results and the analysis of transgenic *Arabidopsis* with a full-length promoter and intron construct showed that the *BnFAD2-C5* promoter promotes high levels of gene expression in the genital organs, especially in seeds. We found that the *BnFAD2-C5* intron indeed has an IME function. The regions or *cis*-elements in the *BnFAD2-C5* promoter required for expression in different tissues were assessed in transgenic *Arabidopsis* using GUS/GFP (β-glucuronidase and green fluorescent protein) as reporter genes and were detected through GUS histochemical analysis and western blotting. Both SA and JA increased *BnFAD2-C5* promoter activity, and the relative response elements were also analyzed in the *BnFAD2-C5* promoter.

Materials and methods

Plant materials

Fifteen *Brassica napus* cv. Xiangyou plants were planted in the field, and *Arabidopsis thaliana* (ecotype Columbia), preserved by our laboratory, was cultivated in an incubator

(BINDER, Germany) under a 16/8-h [light (24 °C)/dark (22 °C)] photoperiod.

Gene cloning

Total DNA was isolated with a DNA extraction kit (TaKaRa, Japan) from 15 Xiangyou leaves. The conserved primer pairs P1/P2 from Xiao et al. (2008) were used to amplify the BnFAD2-C5 gene coding sequence. The PCR products were purified using the DNA gel-extraction kit (TaKaRa, Japan) and then ligated with pMD18-T vector (TaKaRa, Japan) at 4 °C for overnight. Then, the ligation products were transformed into *E. coli* DH5α. Selected ten positive colonies on ampicillin agar plate were sequenced in BoShang Corporation.

To identify the transcription initiation site of the *BnFAD2-C5* gene, the full length of *BnFAD2-C5* cDNA was isolated. Total RNA was extracted with the CTAB method (Chang et al. 1993) from 15 Xiangyou leaves and was then digested with RNase-free DNase (TaKaRa, Japan) at 37 °C for 30 min to remove genomic DNA. The digested total RNA was used to synthesize cDNA using a SMARTerTM cDNA Amplification Kit (TaKaRa, Japan) according to the manufacturer's protocol.

Quantitative RT-PCR to characterize *BnFAD2-C5* gene expression

Total RNA was extracted from 15 Xiangyou roots, leaves, flowers, developing seeds, and the silique coat 20–30 days after flowering (DAF), using the CTAB method. Genomic DNA was digested from the total RNA with an RNase-free DNase (TaKaRa, Japan) at 37 °C for 30 min. cDNA was synthesized using a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's protocol. Quantitative RT-PCR was conducted using YG-C5-FAD2-F/YG-C5-FAD2-R (Table 1) primers and *2ACTIN* as an internal reference gene with the above cDNA as templates. The PCR was performed with a predenaturation at 95 °C for 30 s and 40 cycles of 95 °C for 5 s, and 60 °C for 30 s.

Isolation of the *BnFAD2-C5* promoter and intron

On the basis of the homology between *Brassica napus* and *Brassica oleracea*, the specific primers C5 (pro)-F/C5-FAD2-R were used to amplify the 5′flanking region, including the 1257 bp promoter and the 1123 bp intron, from the genomic DNA of 20 DAF rapeseeds by PCR using HS polymerase (TaKaRa, Japan). The PCR program was 1 cycle of 95 °C for 5 min, 95 °C for 50 s, 56 °C for 50 s, and 72 °C for 3 min; 30 cycles of 95 °C for 50 s, 56 °C for 50 s, and 72 °C for 3 min; and 1 cycle at 72 °C for 10 min. The PCR products were analyzed on 1.5 % agarose gels, and fragments were recovered and purified with a DNA gel-extraction kit (TaKaRa, Japan) and then ligated with pMD18-T vector at 4 °C for overnight. Then, the ligation products were transformed into *E. coli* DH5α. The selected positive colonies were sequenced by the BoShang Corporation.

Construction of the promoter-GUS/GFP fusion vectors and transformation of *Arabidopsis*

To clone the 5′-deletion series of the *BnFAD2-C5* promoter into the binary vector pCAMBIA1303, the 5′flanking region of *BnFAD2-C5* was amplified using the gene-specific primers PBn-R as the reverse primer and PBn-F1, PBn-F2, PBn-F3, PBn-F4, and PBn-F5 as the forward primers. The PCR products were cloned into between the *Hin*dIII and *Nco*I sites of the pCAMBIA1303 vector after purification from the agarose gels and were named constructs PBn-1, PBn-2, PBn-3, PBn-4, and PBn-5.

Table 1 List of primers used in experiments

	Primers	Sequences (5′–3′)
Forward	P1	GCGGCCGCATGGGTGCAGGTGGAAGAATGCAAG
Reverse	P2	GGATCC ACTTATTGTTGTACCAGAACACACC
Forward	YG-C5-FAD2-F	AAAGAACAAAGAAGATATTGTCACG
Reverse	YG-C5-FAD2-R	AAAACAAAGACGACCAGAGACAGCA
Forward	C5(pro)-F	AAATGAAATGAAATCATGGTAGGTG
Reverse	C5-FAD2-R	GCTTGATGTTGTGTCGGTTTCAGACTT
Forward	PBn-F1	CCCAAGCTTGAATGTTACACTAAATTGGATTGGT
Forward	PBn-F2	CCCAAGCTTGCATCTCTAATGTACACTTCTGTAA
Forward	PBn-F3	CCCAAGCTTCTCCAAAGTACACATCACACACACT
Forward	PBn-F4	CCCAAGCTTCAAACTAGTCTTTGTGAACATTAGG
Forward	PBn-F5	CCCAAGCTTTAAAGAAAAGAAATATGAGGAGAGG
Reverse	PBn-R	CATGCCATGGGCTGACGTAGGGGGTGAAGATTTAA
Reverse	IBn-r	CATGCCATGGGTTTCTGCATAAGCCAAAAGCAAAG

Underlined letters represent the HindIII and NcoI sites

The 5′flanking region, including the promoter and intron, was amplified using the primers PBn-F1/IBn-r, and the products were inserted between the *Hin*dIII and *Nco*I sites of the pCAMBIA1303 vector to generate the Bn-1 construct. The pCAMBIA1303 vector was digested with *Hin*dIII and *Sal*I to remove the CAMV35S promoter and create the negative control, and the pCAMBIA1303 vector with the CAMV35S promoter was the positive control. The full length of the *BnFAD2-C5* intron was inserted into the negative control and positive control to generate the Bn-2 and CAMV35S + intron constructs, respectively.

All the constructs were introduced into agrobacterium tumefaciens strain GV3101 via the freeze–thaw method (An 1987). *Arabidopsis* was transformed by the floral dip method (Clough and Bent 1998). A large number of the collected seeds were sterilized in 75 % ethanol for 5 min and then in 26 % sodium hypochlorite for 10 min, and the seeds were then rinsed with sterile water five times. Finally, seeds were germinated on selective medium (4.45 g/l MS, 3 % sucrose, 2.5 g/l phytagar, and pH 6.0) containing 50 mg/l hygromycinb. The plants with well-developed roots were transferred to the soil to obtain the T2 generation.

Exogenous salicylic acid and jasmonic acid application

The T2 generation seeds of BnP-1, BnP-2, and BnP-4 were selected with MS medium with hygromycinb. After 7 days, the positive seedlings of BnP-1 and BnP-2 were treated with salicylic acid, and the positive seedlings of BnP-4 were treated with jasmonic acid. All the seedlings were treated with various hormone concentrations for 24 h under a 16/8-h photoperiod [light (24 °C)/dark (22 °C)].

Histochemical analysis of GUS and western blotting

The roots, stems, leaves, flowers, and developing seeds were cut and incubated immediately in GUS staining buffer [1 M NaH_2PO_4, 0.5 M Na_2HPO_4, 0.1 M K3[Fe(CN)6], 0.1 M K4[Fe(CN)6]$\cdot$3H_2O, 0.5 M Na_2EDTA(pH 8.0), 5 % methanol, 0.12 % TritonX-100, and 0.1 mM X-Gluc] for 16 h at 37 °C. The stained tissues were then rinsed with 70 % ethanol until the chlorophyll had been cleared completely, and the images were photographed using an SZX2-ILLB stereomicroscope (Olympus, Japan).

The green fluorescent protein (GFP) in the pCAMBIA1303 vector after GUS was quantitatively detected by western blotting. Total protein was extracted from the T2 generation transgenic *Arabidopsis thaliana* roots, stems, leaves, flowers, and seeds according to Noir et al. (2005). An 8 μl protein sample was mixed with 2 μl of 5 × SDS-PAGE sample loading buffer (Beyotime, China) and was preheated for 10 min at 65 °C to denature the protein completely, and samples were loaded onto SDS-PAGE gels. The SDS-PAGE gels were composed of 10 ml of 12 % separating gel and 5 ml of 3 % stacking gel, and the electrophoresis procedure was 180 V for 1.5 h and then 80 V for 1.5 h using a Bio-Rad electrophoresis apparatus. Afterward, the protein was transferred into a 0.45 μm PVDF membrane by trans-membrane electrophoresis. Then, the membrane with the protein of interest was incubated with blocking buffer (5 % skim milk powder solution) for 1 h, washed, incubated for 1 h with primary antibody GFP (Beyotime, China), and washed 5 times, incubated with a secondary antibody (HRP-labeled goat anti-mouse IgG) for 1 h, and subjected to a final wash step. The processes of blocking primary and secondary antibody incubation were all performed on a shaking Table (54 rpm). The last step was the detection of the protein. ECL luminescence solution was added onto the PVDF membrane, which was then placed under an X-ray film for 5 min. The X-ray film was then developed for 2 min and then fixed for 2 min. After drying of the X-ray film, it was scanned with a GS-800 Calibrated Densitometer, and the GFP content was calculated with the Quality One software.

Results

Expression analysis of the *BnFAD2-C5* gene

At the beginning of our study, the sequencing of the *Brassica napus* genome had not been completed. Homologous cloning and cRACE technology were used to clone the coding sequence (CDS) and the 5′ and 3′ untranslated regions (UTR) of the *BnFAD2-C5* gene, to characterize its expression and regulation patterns. As shown in Fig. 1, the *BnFAD2-C5* gene comprises a 1155 bp open reading frame (ORF) that encodes 384 amino acids, a 1150 bp intron from position +146 to position +1269 within the 5′UTR, and a 351 bp 3′UTR downstream of the CDS.

The expression pattern of the *BnFAD2-C5* gene was obtained by quantitative RT-PCR with a pair of specific primers, which were designed on the basis of the sequences of the 3′UTR region. The *BnFAD2-C5* gene was primarily expressed in the genital organs of *Brassica napus*, including the flower and seed (Fig. 2a). Then, we constructed a vector with a full-length promoter and a full-length intron inserted in front of GUS/GFP and transformed *Arabidopsis*

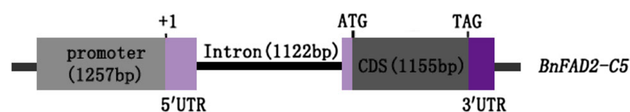

Fig. 1 Structure of *BnFAD2-C5* gene in rapeseed genome

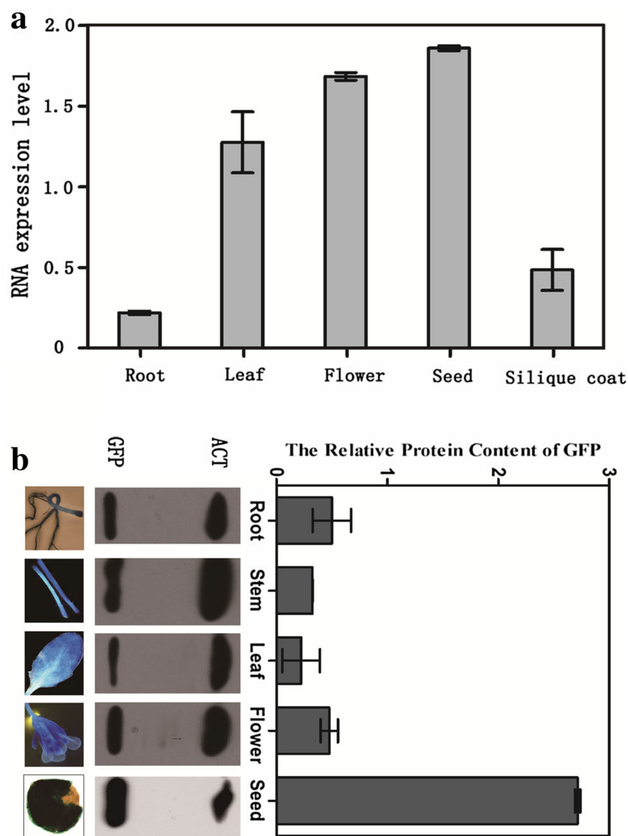

Fig. 2 Assay of expression pattern of *BnFAD2-C5* gene. Quantitative of BnFAD2-C5 gene (**a**) and GFP expression in transgenic *Arabidopsis* with construct Bn-1 which involves the full length of promoter and intron (**b**). Western blot was used for the quantitative analysis of GFP protein. In this progress, ten independent transgenic lines (T2) were examined. The highest and lowest ones were excluded in our data analyses. The values are average ± error

plants to analyze the expression pattern of the *BnFAD2-C5* gene in the T2 transgenic *Arabidopsis* generation. As shown in Fig. 2b, the expression of the reporter gene *GFP* in seeds was much higher than that in other organs, including the root, stem, and leaf. This result was consistent with the results of the histochemical staining. The seeds with the target gene were stained more deeply, and the staining of the root, stem, leaf, and flower was similar. On the basis of these results, we concluded that *BnFAD2-C5* promoter promotes high levels of expression in the genital organs, especially in the seeds.

Sequence analysis of the promoter region of the *BnFAD2-C5* gene

Transcriptional regulation is key to gene expression, and the promoter is a crucial factor in transcriptional regulation. A 1257 bp fragment of the promoter-like region of *BnFAD2-C5* was amplified. Subsequently, the promoter sequence of *BnFAD2-C5* was analyzed for known *cis*-acting elements through a web search of publicly available databases (http://www.dna.affrc.go.jp/htdocs/PLACE and http://www.intra.psb.ugent.be:8080/PlantCARE). Putative TATA-box and CAAT-box elements were found to be located at −139 and −61 bp, respectively.

Through this database analysis, many *cis*-elements were identified, including a Dof core, an E-box, a salicylic acid-responsive element (TCA motif), a methyl jasmonate-responsive element (CGTCA motif), abscisic acid-responsive elements (ABRE, MYB, and MYC), root hair specific elements (ARS element and RHE), an anoxic-responsive element (GC motif), and light regulation elements (Box I, Box II, Box III, Box IV, GAG, G-box, and GT-1 consensus). Furthermore, AGAAA was involved in pollen-specific expression, GATA was required for high-level, light dependent, and tissue-specific expression, and ACGT was required for the endosperm-specific expression and the 5′UTR Py-rich stretch that conferred high transcription levels (Fig. 3).

In many plants, such as cotton (Pirtle et al. 2001), soybeans (Heppard et al. 1996), sesame (Kim et al. 2006), oilseed (Xiao et al. 2014), and rice (http://www.cdna01.dna.aVrc.go.jp/cDNA), the *FAD2* genes have a large intron within the 5′-UTR. We also found an 1123 bp-length fragment of the intron in the *BnFAD2-C5* gene. This intron had 43.3 % T content, 25.9 % A content, and GT-AG dinucleotides at both ends, which are well-known characteristics of higher plant introns. The intron sequence was also evaluated for known *cis*-acting elements through a web search of PLACE and PlantCARE. The promoter-like *cis*-elements, the TATA-box element, and CAAT-box element were at positions +1241 and +1040, respectively. Several potential *cis*-elements, including ACGT, MYB, MYC, Dof, WRKY, HSE, G-box, E-box, GARE, CAT box, TC-rich, and the GT-1 consensus, were predicted to be located within that region (Fig. 3). Although the promoter and the intron shared a number of sequence elements, the overall sequence identity was only 37.5 %, thus revealing that the promoter and intron did not arise from tandem replication.

The *BnFAD2-C5* intron is involved in intron-mediated enhancement (IME)

Kim et al. (2006) and Xiao et al. (2014) have reported that, in sesame and oilseed, respectively, the single intron sequence slightly activates the activity of the promoter. Thus, to characterize the *BnFAD2-C5* intron, we cloned the intron sequence, including the short 5′UTR (137 bp), inserted it into a pCAMBIA1303 vector to construct Bn-3, and deleted CAMV35S from the pCAMBIA1303 vector to construct the negative control. The Bn-3 construct and the negative control construct were transformed into

```
-1257  GAATGTTACA CTAAATTGGA TTGGTTTTCA ACTTTCACAA ATAAAAAGTA CTATTTATAA AATTAGAAAA AAATATATCA
                CACT                                          Dof                   pollen/ GT-1consensus
-1177  AGACTATTCT TTTTTAGAGG AAGAAATAGA AGAATACATT GGAAACAAAT CTATCTCTAT TATATAGTTT TCCTATTTTA
                             pollen/BoxII / TCA motif
-1097  GAAAAAAAAA ATAGAGAAAT ACATTGGAGA TGGTTTAAGC GGTAGTAACA CAAAGAAAAA CTCTAAATAT CTTAAGAGCA
       GT-1consensus                GAG                          Dof / GT-1consensus
-1017  TCTCTAATGT ACACTTCTGT AATTTCTTCT AAAATAGAGA TCTCTATTAT AGAGGTGAAA ATGCTCCAAT GTATGCCTCT
                  CACT                                          GTGA / GT-1consensus  CCAATbox
-937   ATAATAGAAT TCATCTATTT TAAAAGAAAA TATAGAGAAA AATTACTTTT TGCTTTTATA TTTAAAGGTG GAAATAAAAT
         BoxII               Dof / GT-1consensus  GT-1consensus      ARS element / SEF1 / Dof  GT-1consensus
-857   ATCTCTATAT AAATAAATAA ACTCTATTAT ACATGTATAC ATTGGAGCAT TTTCACTTTT ATAATAGAGT TTTTTTATTT
                                                   BoxIII / CACT                  T Box
-777   TAAGAAAAAA TATAGAGATA GAAATAGAAA TAGAAATAGA GATGAGTTGG AGATTAGAAA TAGAGATGAG TTTGAGATGT
       GT-1consensus        GATA / 3BoxII           GAG                              GAG
-697   TGTTACGTAA GAAAGAGCTA GAGCTTTAAT AAAGTACTTA AATTAATTAC TAGTCGGCAG TCGCTGCCTA CTTGTTTACC
           ACGT    Dof                  Dof        Box4
-617   ACCTAAATTA ATTTATTATA ATATATATTA CGAATCTCCA AAGTACACAT CACACACACT CTACTCACGT GATCTCAACC
         Box4                                 Dof                CACT  ABRE / G-box / GTGA
-537   ACAATGTCTG CAGATATTTT TTATAGTTTT TTCTCACATG GGAGAGAAGA AGCCAAGCAC GATCCTCCAT CCTCAACTTT
                                        E-box / MYC                   RHE
-457   ATAGCATTTT TTTCTTTTCT TTCCGGCTAC CACTTGTGAG TCGAGTCGGC AAGGGCGTTT CCTTATATTA AAGTAAAGAC
              5' UTR Py-rich strech    CACT / E-box / MYC / GTGA                                 Dof
-377   ATCAAATACC ATCGTCTTAA TGCTAATTAA CGTAATTGAT GAGTTCTATA ACATAATCCA AACTAGTCTT TGTGAACATT
                                                                                   GT-1 consensus
-297   AGGATTGGGT AAACCAATAT TTACATTTTA AAAACAAAAT ACAAAAAGAA ACGTGATAAA CTTTATAAAA GCAATTATAT
                  MYB                  ANAER01        Dof pollen/G-box / ABRE / GT-1consensus Dof
-217   GATCACTGCA TCTTTTCCAC TTTTCCGTAA ATAAATACAT AAAAGTGCCG TAAATATCAG ATATTTGGAG TAGAAAAGTA
          CACT         CACT                        Dof                  GATA         2GT-1consensus /
-137   ATAAAGAAAA GAAATATGAG GAGAGGGAAT AATGGAGGGG GCCCACTTGT AAAAAAGAAA GAAAAGAGAT GTCACTCAAT
       3Dof / pollen/TATAbox                                  Dof / GT-1consensus          CAATbox
-57    CGTCTCCCAC GGGCCCCCGT CAATTTAAAC GGCCTGCCTT CTGCCCAATC GCATCTTATC AGAACCAGAC AGATTCATTA
                  GC-motif / CGTCA motif   myb                                +1
+24    CCAAAGAGAT AGAGAAAGAG AGAGAGAGAG AGAGAGAGAG AGAGTGAGTT TGAGGAGGAG CTTCTTCGTA GGGTTCATCG
+104   TTATTAACGT TAAATCTTCA  CCCCCTACGT CAGCCAGCTC AAGgtccctt tcttcttcca tttcttttca ttctacgttg
                                                                                        ACGT
+184   ttttcaatct tatgaaactt tctggtctgt gcttttctta tcgcttttct atttcatcta tcatttttgc atttcagtcg
+264   atttaattct agatctgtta atattaaact atagatctgt tcttgattct ctgttttcat gtgtgaaatc tgatgctgta
                     MYB core                                        Ebox / GTGA
+344   ttaatctgat tatattgtct ataccgtgga gaatatcaaa tgttgcattt tcatttgtcc gaatacaaag tgtttgactt
                                              Ebox / MYC     Ebox / MYC     Dof     WRKY
+424   tcaatcgttt ttaattatat atatatatat attttttgat gggttggtgg agttgaaaaa tcaccatagc agtctcacgt
                                                          GT-1consensus              G-box / ACGT
+504   cctggtttta gaaatatcct attcaaaatt atatatttgt ttacttgttt tagatctgga cctgagacat ataagtacct

+584   atttgttgaa tctttgggta aaaacttatg tctctgggta aaatttgctg ggagatttga ccgattccta ttggctcttg
                     GT-1consensus         GT-1consensus          WRKY
+664   attctgtagt tacgtaatac atgaaaaagt ttcatttggc ctatgctcac ttcatgctta taaacgtttt cttgcaaatt
                  ACGT     HSE / GT-1consensus / Dof / Ebox / MYC          ACGT / TC-rich
+744   aattggatta gatgttattt catagattca gtcattcaga tacaatggag ttgcatgaag aaaataatag aattcgtgac
                                       skn-1motif   GATA                       BoxII / WRKY  GTGA / WRKY
+824   agtaaaaaag attgtatttt  tgtttgtttg tttatgttta aaagtctata tgttgacaat agagttgctc tcaactgttt
            Dof                                   Dof       WRKY / Wbox           Ebox / MYB / MYC
+904   catttagctt cttttttgt caagttgctt attcttagag acattgtgat tatgacttgt cttctttaac gtagtttagt
                                                     GTGA / Wbox / WRKY       GARE / ACGT
+984   aataaaagac gaaagaaatt gatatccaca agaaagagat gtgagctgta gcgtatcaaa tctcgttcat ttactagtag
         Dof        Dof      GTGA      Dof / GAGmotif  GTGA       CAAT box
+1064  tattctcaac gctatcgttt atttattttt ctttcgttgg tttgccacta tatgccactt ctctcctctt tgtcccacgt
                                                    CAT-box    CAT-box                  ACGT
+1144  actatccatt ttttttgtgg tagtccattt tcttgtaact tataataacg taactctgaa tcttttgtct gtagattaat
       G-box / CACT / Box2 / TCA-element               ACGT
+1224  ttgttggttt aattaacttt taagtctttg cttttggctt atgcagAAAC
                      TATA-box
```

◀**Fig. 3** Nucleotide sequences of the 5′ flanking region and intron of *BnFAD2-C5*. A *black box* represents the transcription initiation site identified by 5′-RACE and is designated as the +1 position; the numbering on the *left* refers to nucleotide sequences; the sequences of the *BnFAD2-C5* intron are shown in *lowercase letters*; several potential *cis*-elements are *underlined* and designated with the names of each of the motifs

Arabidopsis and were detected in various tissues in the T2 generation, including the root, stem, leaf, flower, and seed, through histochemical staining. However, the results of the staining showed no differences between the Bn-3 construct and the negative control construct (Fig. 4a), thus indicating that the *BnFAD2-C5* intron does not serve as a promoter, even though it has putative promoter-like elements as determined through bioinformatics.

When the intron was inserted after the *BnFAD2-C5* promoter, the GFP expression was approximately fourfold higher than that of the construct with only the promoter sequence, thus suggesting that the intron improved the expression level of the *BnFAD2-C5* gene (Fig. 5a). For the construct with the intron inserted downstream of the CAMV35S promoter, as compared with the construct with only the promoter CAMV35S, the expression levels in the root, stem, leaf, and even the flower decreased, but the GFP expression was approximately 1.6-fold higher in the seed (Fig. 5b).

All the results revealed that the *BnFAD2-C5* intron is involved in intron-mediated enhancement (IME). Moreover, the *BnFAD2-C5* intron served as its own promoter in various transgenic *Arabidopsis* tissues but promoted the exogenous CAMV35S promoter only in the seeds of transgenic *Arabidopsis*.

The *BnFAD2-C5* gene is expressed in various tissues

To define the regulatory sequences that control the expression of the *BnFAD2-C5* gene, a series of promoter deletions (BnP-1, BnP-2, BnP-3, BnP-4, and BnP-5) fused to a GUS/GFP reporter gene were constructed on the basis of the bioinformatics analysis (Fig. 3). The deletion constructs included the short 5′UTR (137 bp) and were transformed into the wild *Arabidopsis*. Up to ten independent primary transformants were regenerated for each construct until the T2 generation. None of the tissues of the transgenic *Arabidopsis* plants with the BnP-5 promoter fragment could be stained, thus suggesting that this region does not play the role of the promoter and that the BnP-4 is the minimal promoter (Fig. 6a).

The GUS histochemical staining showed that the transgenic *Arabidopsis* root with the BnP-2 promoter fragment exhibited the strongest staining (Fig. 6b), and the data analysis indicated that the highest level in the transgenic *Arabidopsis* root was with the BnP-2 promoter fragment (Fig. 6d). Furthermore, when the −1020 to −581 and −581 to −319 bp regions were deleted, the GFP activity of the root decreased. It is possible that the region of the −1020 to −319 bp promoter fragment is the main control reporter gene expressed in the root. It is also possible that this promoter fragment primarily controls the *BnFAD2-C5* gene expression in the root. As shown in Fig. 6d, when the −1020 to −581 bp region was deleted, the relative content of GFP substantially decreased in the transgenic plants' stem, thus suggesting that this region maintains the gene expression in the stem, although the histochemical staining was not obvious. In the transgenic *Arabidopsis* leaves with the BnP-3 promoter fragment, the relative content of GFP was the highest (Fig. 6d), and this corresponded to the staining results (Fig. 6b); however, this content decreased when the −581 to −319 bp region was deleted, thus suggesting that the −581 to −319 bp region primarily controls the expression of the *BnFAD2-C5* gene in the leaf. When the −1257 to −1020 bp region was deleted to create construct BnP-2, the relative content of GFP decreased substantially in the flowers of the transgenic *Arabidopsis*, thus suggesting that the −1257 to −1020 bp region

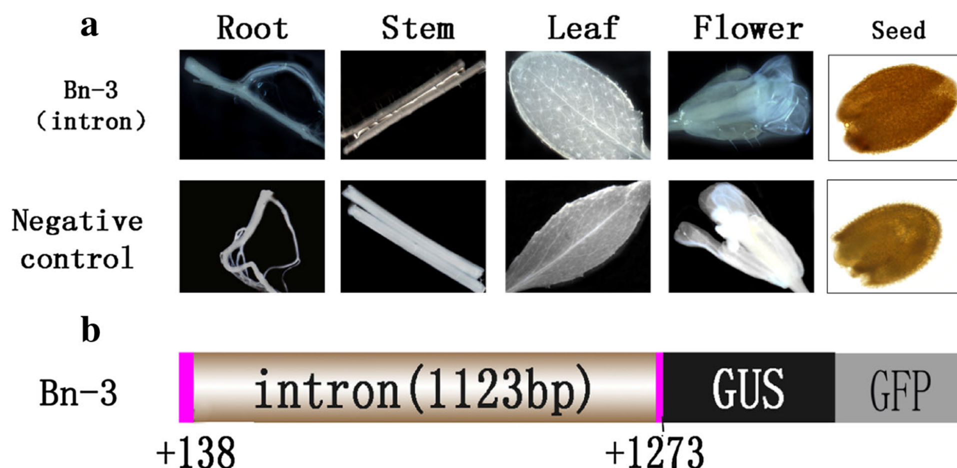

Fig. 4 Function analysis of *BnFAD2-C5* intron. **a** GUS histochemical analysis of Bn-3 construct and negative control construct; **b** Scheme figure of Bn-3 construct

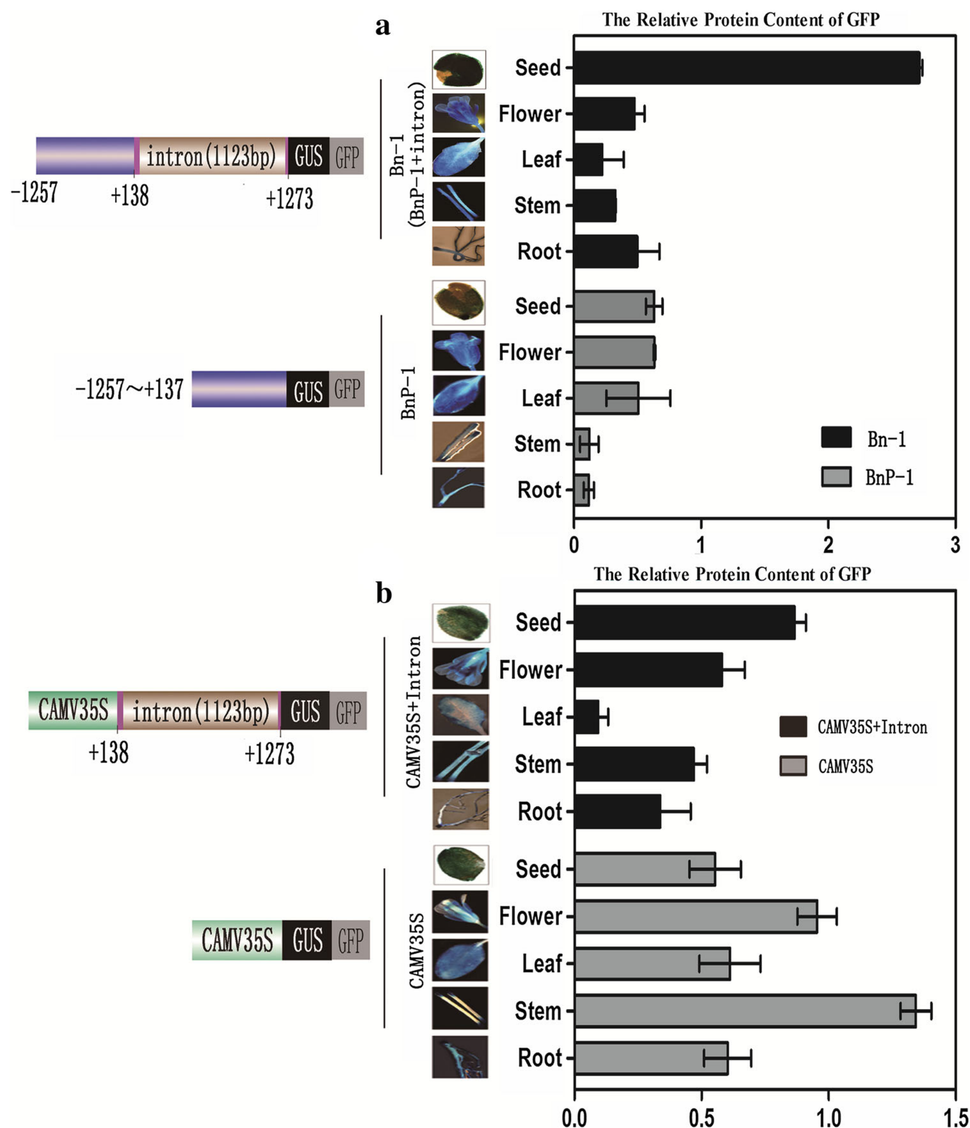

Fig. 5 *BnFAD2-C5* intron analyses in transgenic *Arabidopsis*. **a** Comparative analyses of GFP in various tissues between the construct with *BnFAD2-C5* intron downstream after itself promoter or not using histochemical and quantitative (western blot) methods. **b** Comparative analyses of GFP in various tissues between the construct with *BnFAD2-C5* intron downstream after CAMV35 promoter or not using histochemical and quantitative (western blot) methods. The values are average ± standard error

probably controls the expression of the gene in floral organization. However, the relative content of GFP in the transgenic *Arabidopsis* flower with the BnP-3 and BnP-4 promoter fragments increased, possibly because of the deletion of the negative *cis*-elements in the −1020 to −581 bp region, which might have inhibited *BnFAD2-C5* gene expression in the flower.

Notably, the expression of the *BnFAD2-C5* promoter was active in the root, stem, leaf, flower, and especially in the seeds of the transgenic *Arabidopsis* with a series of deleted promoter fragments. As shown in Fig. 6b, the GUS histochemical staining decreased in the transgenic plants' seeds from the BnP-1 to the BnP-3 construct, but this effect depended on the BnP-4 construct. This result was consistent with the analysis of the GFP content. The deletion from −1257 to −581 bp decreased the GFP content, thus suggesting that the −1257 to −581 bp region contains some *cis*-elements, which improves gene expression. However, when the −581 to −319 bp region was deleted, the expression level of GFP increased markedly (Fig. 6c). This result suggested that some negative *cis* elements exist in the −581 to −319 bp region. As shown in Fig. 6c, the GFP expression in the transgenic *Arabidopsis* seeds with the −319 to −1 bp promoter fragment was higher than that with the full promoter, thus suggesting that −319 to −1 bp region is the main region driving expression.

Exogenous salicylic acid and jasmonic acid application changes *BnFAD2-C5* promoter activity

The bioinformatics analysis results showed that some hormone responsive *cis*-elements were present in the *BnFAD2-C5* promoter region. To verify these results and to determine whether hormonal control affects the

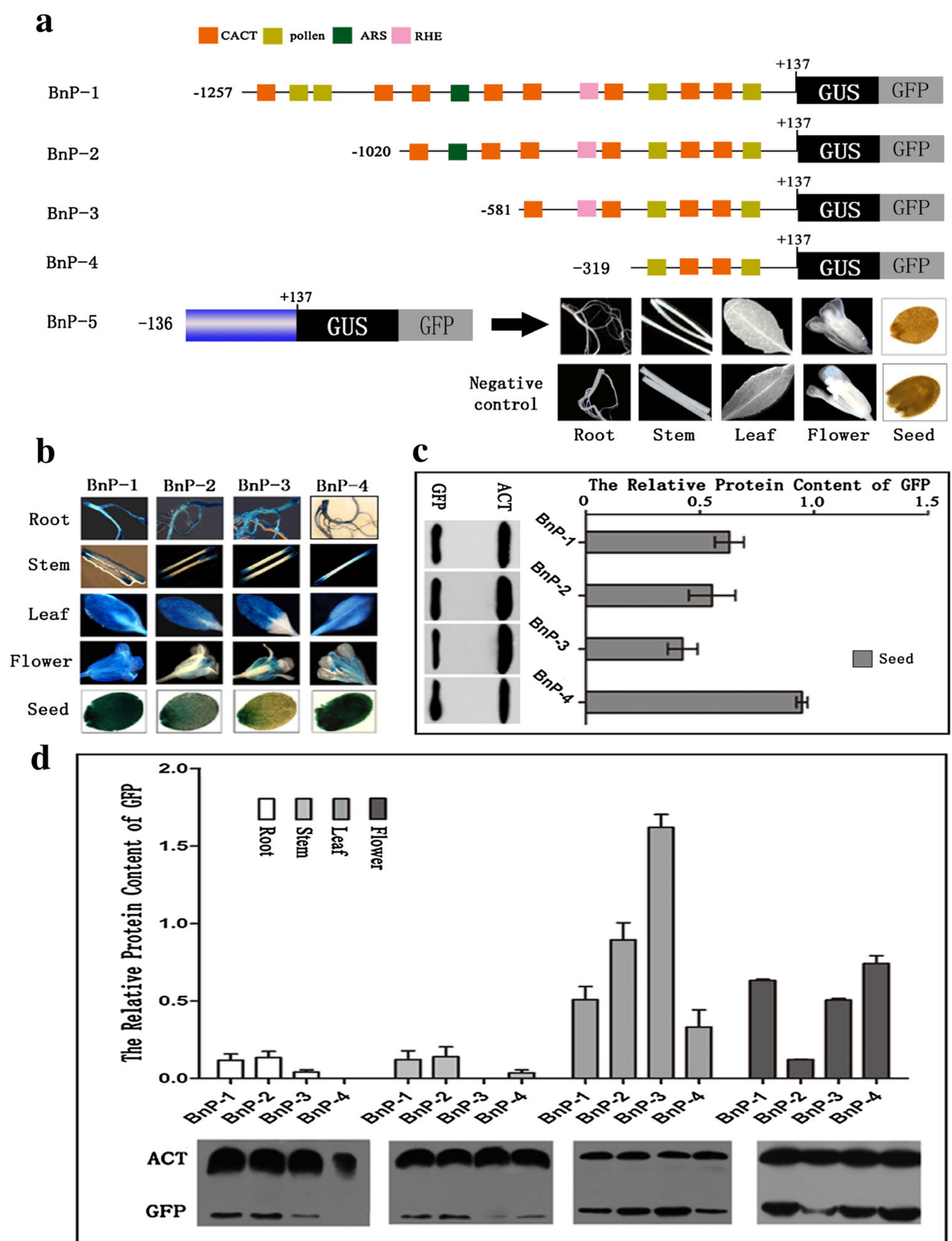

Fig. 6 *BnFAD2-C5* promoter analyses in transgenic *Arabidopsis*. Histochemical (**a**, **b**) of GUS and quantitative (**c**, **d**) of GFP expression in transgenic *Arabidopsis*. In histochemical staining and GFP quantity analyses measurements, ten independent transgenic lines (T2) were examined for each construct. The values are average ± standard error. The seed was transgenic *Arabidopsis* developing seeds between 7 and 10 DAF. **a** Model diagram of deleted promoter fragments with different hormone responsive elements

expression of the *BnFAD2-C5* promoter, the T2 generation seedlings, with the relevant promoter fragments, were incubated with various concentrations of salicylic acid (SA) and jasmonic acid (JA), which were predicted to have response elements in the promoter. As shown in Fig. 7, when the seedlings with the BnP-1 promoter fragment containing the TCA element, which responded to SA, were treated with various SA concentrations, the relative content of GFP detected by western blotting increased. However, when the TCA element was deleted (BnP-2, the relative content of GFP did not show obvious changes). Thus, the −1257 to −1020 bp region harbors the SA-response element. Moreover, the seedlings with the BnP-4 promoter fragment containing the CGTCA motif, which responded to JA, were treated with various JA concentrations, and western blot analysis revealed that an exogenous JA application resulted in an approximately 3—5-fold increase in the expression of the reporter gene. These results indicated that the −319 to −1 bp region harbors the JA-response element. All these results enabled us to delineate the region harboring an SA-response element and the region harboring a JA-response element. Furthermore, we predict that these two elements exist at −1150 to −1141 and −39 to −35 bp, respectively.

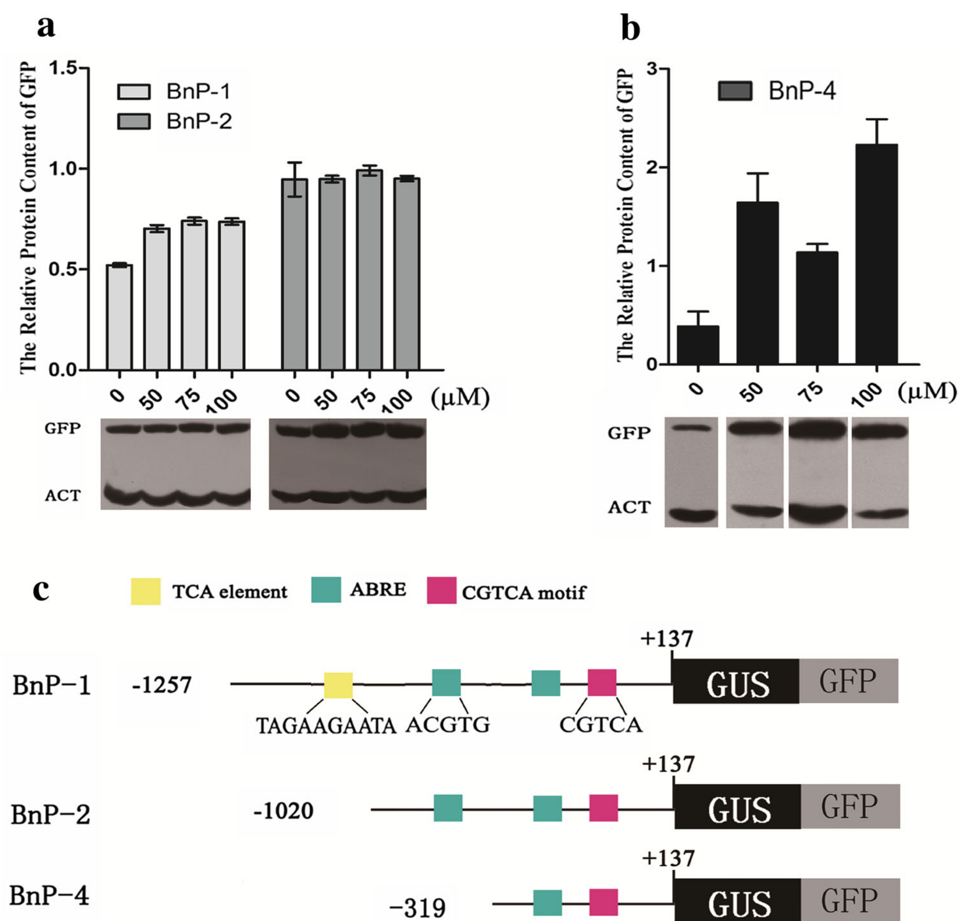

Fig. 7 Quantitative analyses of GFP in transgenic *Arabidopsis* seedlings after the application of SA (**a**) and JA (**b**). **a** Developing transgenic Arabidopsis seedlings was incubated with 0, 50, 75, and 100 μM for 48 h, then subjected to the western blot analysis of GFP gene. **b** Dealt with a serious concentration of JA (0, 50, 75, and 100 μM) for 48 h and detected as (**a**). **c** Model diagram of different deleted promoter fragments

Discussion

The functions of the *FAD2* copies in *Brassica napus*

Four *FAD2* copies have been identified and located in an N1 linkage group (*BnFAD2-A1* and *BnFAD2-C1*) and an N5 linkage group (*BnFAD2-A5* and *BnFAD2-C5*) in *Brassica napus* (Scheffler et al. 1997; Smooker et al. 2011; Yang et al. 2012; Lee et al. 2013). Each *FAD2* copy is an independent gene, and the cooperation among copies maintains normal development. Thus, studying the function of each *FAD2* copy is important.

Of the four copies, only *BnFAD2-C1*, *BnFAD2-A5,* and *BnFAD2-C5* play a role in fatty acid desaturation and function to different degrees. The *BnFAD2-A5* and *BnFAD2-C5* genes contribute much more than *BnFAD2-C1* (Lee et al. 2013). *BnFAD2-C5* is highly expressed in the flower especially in the seed (Fig. 2), whereas *BnFAD2-C5* primarily regulates and controls expression in genital organs.

An intron within the 5′UTR is involved in IME function

The previous analysis showed that the single *BnFAD2-C5* intron did not have a promoter function, even though it harbored promoter-like elements (Figs. 3, 4). However, when the intron was inserted downstream of the *BnFAD2-C5* promoter and the *CAMV35S* promoter, it improved the gene expression level significantly (Fig. 5). Namely, the *BnFAD2-C5* intron improved expression from not only the respective promoter but also the exogenous promoter CAMV35S. An intron with an enhanced effect that is widely distributed in the 5′UTR sequence has been identified in maize (Maas et al. 1991), *Arabidopsis* and sesame (Kim et al. 2006). The IME function may be a result of the intron splicing effects (Mascarenhas et al. 1990), the functions of specific elements in specific regions of the intron interacting with the promoter regions (Kim et al. 2006) or the length of the intron serving as a spacer (Chung et al. 2006). The phenomenon in which the *BnFAD2-C5*

intron enhances the CAMV35S promoter activity may be a result of the above three reasons and requires further study.

The intron with an IME typically contains a U-rich sequence, and our analysis indicated a 38.7 % U sequence in this region as well as an ABRE/MYB/MYC, E-box, Wbox, and GARE, which promote high-level expression via the corresponding promoter in rapeseed and *Arabidopsis* (Stålberg et al. 1996; Eulgem et al. 2000). An E-box usually appears with some hormone responsive elements, such as ABRE/MYB/MYC (Liu et al. 2015; Lenka et al. 2015), thus providing a site for bHLH proteins to bind and consequently regulate gene expression. The WRKY/W box is usually stimulated by a pathogen or salicylic acid (SA), and it plays a defense function in plant defense-related genes (Dong et al. 2003). Overall, the elements in this intron are mostly hormone and defense responsive elements, which are crucial factors affecting plant physiology. Thus, at specific stages of growth, the internal hormones produced by plants might stimulate high expression of the *BnFAD2-C5* gene. However, this possibility remains to be verified by additional experiments.

Different regions are responsible for the *BnFAD2-C5* gene expression in various tissues

Although most oil existed in rape seeds used for oil manufacture, it also distributes in other organizations, such as root, stem, leaf, and flower. We detected *BnFAD2-C5* expression not only in the seeds but also in other organs. Transcriptional regulation plays an important role in gene expression, and the promoter is crucial in this process. Here, we demonstrated that the −1020 to −319 bp region harbors ARS and RHE elements, which regulate root-specific gene expression. These elements are located in the −1020 to −581 and −581 to −319 bp regions, respectively. This finding may explain why the expression in the root decreased from BnP-2 to BnP-3, and even to BnP-4. Several CACT elements, which are key elements of the mesophyll expression module (Gowik et al. 2004), are distributed along the full length of the *BnFAD2-C5* promoter. The decreased expression of GFP in the leaf with BnP-4 may have been caused by the deletion of most of the CACT in the −1257 to −319 bp region. Another important element regulating gene expression in the flower is the pollen element (Dzelzkalns et al. 1993). Two pollen elements exist in the −1257 to −1020 bp region, and another two are in the BnP-4 construct. Thus, the expression of the reporter gene was high in the flower with the BnP-1 construct, but it decreased approximately fourfold after the deletion of the −1257 to −1020 bp region in the BnP-2 construct. However, the expression in the flower with the BnP-4 construct was as high as that with BnP-1, probably because some negative *cis*-elements that inhibit the function of pollen element in BnP-4 were deleted.

Although the *BnFAD2-C5* promoter is not the seed-specific promoter, it contains some elements that can activate the promoter in seeds. In the −319 to −1 bp region, there are 4 Dof, GAG, GC-motif, G-box, CGTCA motif, 3GT-1 consensus, GATA, TATA-box, and CAAT-box *cis*-elements. The GT-1 consensus has been identified to promote SCaM-4 gene expression in plants infected by pathogens or treated with NaCl (Park et al. 2004). The Dof and GAG are induced by light, and when they are stimulated by light, the cyPPDK and PEPC genes have high transcription level in maize (Yanagisawa 2000). When the jasmonic acid content increases in the barley plant, the CGTCA motif associates with it and induces high expression of the lipoxygenase1 gene in the barley grain (Rouster et al. 1997). The GATA element strongly improves the transcriptional level of the cab promoter (Lam and Chua 1989), and the CAAT-box mediates the gene expression level by increasing the frequency of transcription initiation (Kusnetsov et al. 1999). Overall, these *cis*-elements may improve gene expression after stimulation with the relevant factor.

SA, ABA, and JA induce the expression of *BnFAD2-C5*

The *Brassica napus* genome originated from *Brassica oleracea* (CC) and *Brassica rapeseed* (AA), and high homology exists between *B. oleracea* and *B. rapeseed*. The alignment of the 5′flanking sequences between *BnFAD2-A5* and *BnFAD2-C5* in *Brassica napus* showed that the highly homologous regions of the promoter were mainly in the 600 bp next to the transcription initiation site (data not shown). We have identified the existence of the ABRE in the *BnFAD2-A5* promoter region, which is homologous to the *BnFAD2-C5* promoter, as published previously (Xiao et al. 2014). The *BnFAD2-C5* promoter also functions with the ABA for its ABRE. We found that a TCA element and the CGTCA motif are responsible for SA and JA responses. These findings may provide some clues as to how to increase the content of oleic acid to improve the quality of oil.

Acknowledgments This work was supported by the Graduate Innovation Foundation of Hunan (CX2013A012) and the Major State Basic Research Development Program of China (2015CB150200).

References

An G (1987) Binary ti vectors for plant transformation and promoter analysis. Methods Enzymol 153:292–305

Baud S, Lepiniec L (2010) Physiological and developmental regulation of seed oil production. Prog Lipid Res 49:235–249

Bäumlein H, Miséra S, Luerßen H, Kölle K, Horstmann C, Wobus U, Müller AJ (1994) The *FUS3* gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. Plant J 6:379–387

Beisson F, Koo AJ, Ruuska S, Schwender J, Pollard M, Thelen JJ, Ohlrogge JB (2003) Arabidopsis genes involved in acyl lipid metabolism. A census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. Plant Physiol 132:681–697

Bilyeu KD, Palavalli L, Sleper DA, Beuselinck PR (2003) Three microsomal omega-3 fatty-acid desaturase genes contribute to soybean linolenic acid levels. Crop Sci 43:1833–1838

Bolle C, Herrmann RG, Oelmüller R (1996) Intron sequences are involved in the plastid-and light-dependent expression of the spinach *PsaD* gene. Plant J 10:919–924

Callis J, Fromm M, Walbot V (1987) Introns increase gene expression in cultured maize cells. Genes Dev 1:1183–1200

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Chen Y, Zhou XR, Zhang ZJ, Dribnenki P, Singh S, Green A (2015) Development of high oleic oil crop platform in flax through RNAi-mediated multiple *FAD2* gene silencing. Plant Cell Rep 34:643–653

Chung BY, Simons C, Firth AE, Brown CM, Hellens RP (2006) Effect of 5′UTR introns on gene expression in *Arabidopsis thaliana*. BMC Genom 7:120

Clancy M, Hannah LC (2002) Splicing of the maize Sh1 first intron is essential for enhancement of gene expression, and a T-rich motif increases expression without affecting splicing. Plant Physiol 130:918–929

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Deyholos MK, Sieburth LE (2000) Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. Plant Cell Online 12:1799–1810

Dong J, Chen C, Chen Z (2003) Expression profiles of the *Arabidopsis WRKY* gene superfamily during plant defense response. Plant Mol Biol 51:21–37

Dzelzkalns VA, Thorsness MK, Dwyer KG, Baxter JS, Balent MA, Nasrallah ME, Nasrallah JB (1993) Distinct *cis*-acting elements direct pistil-specific and pollen-specific activity of the Brassica S locus glycoprotein gene promoter. Plant Cell 5:855–863

Eulgem T, Rushton PJ, Robatzek S (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5:199–206

Finkelstein R, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell Suppl 14:S15–S45

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Gowik U, Burscheidt J, Akyildiz M, Schlue U, Koczor M, Streubel M, Westhoff P (2004) *cis*-Regulatory elements for mesophyll-specific gene expression in the C4 plant *Flaveria trinervia*, the promoter of the C4 phosphoenolpyruvate carboxylase gene. Plant Cell 16:1077–1090

Guan M, Li X, Guan C (2012) Microarray analysis of differentially expressed genes between *Brassica napus* strains with high- and low-oleic acid contents. Plant Cell Rep 31:929–943

Heppard EP, Kinney AJ, Stecca KL, Miao GH (1996) Developmental and growth temperature regulation of two different microsomal [omega]–6 desaturase genes in soybeans. Plant Physiol 110:311–319

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol 59:41–66

Jin UH, Lee JW, Chung YS, Lee JH, Yi YB, KimY K, Chung CH (2001) Characterization and temporal expression of a ω-6 fatty acid desaturase cDNA from sesame (*Sesamum indicum* L.) seeds. Plant Sci 161:935–941

Jung JH, Kim H, Go YS, Lee SB, Hur CG, Kim HU, Suh MC (2011) Identification of functional BrFAD2-1 gene encoding microsomal delta-12 fatty acid desaturase from *Brassica rapa* and development of *Brassica napus* containing high oleic acid contents. Plant Cell Rep 30:1881–1892

Kim MJ, Kim H, Shin JS, Chung CH, Ohlrogge JB, Suh MC (2006) Seed-specific expression of sesame microsomal oleic acid desaturase is controlled by combinatorial properties between negative *cis*-regulatory elements in the *SeFAD2* promoter and enhancers in the 5′-UTR intron. Mol Genet Genomics 276:351–368

Kusnetsov V, Landsberger M, Meurer J et al (1999) The assembly of the CAAT-box binding complex at a photosynthesis gene promoter is regulated by light, cytokinin, and the stage of the plastids. J Biol Chem 274:36009–36014

Lam E, Chua NH (1989) ASF-2: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in Cab promoters. Plant Cell Online 1:1147–1156

Lee KR, Sohn SI, Jung JH, Kim SH, Roh KH, Kim JB, Kim HU (2013) Functional analysis and tissue-differential expression of four *FAD2* genes in amphidiploid *Brassica napus* derived from *Brassica rapa* and *Brassica oleracea*. Gene 531:253–262

Lenka SK, Nims NE, Vongpaseuth K, Boshar RA, Roberts SC, Walker EL (2015) Jasmonate-responsive expression of paclitaxel biosynthesis genes in *Taxus cuspidata* cultured cells is negatively regulated by the bHLH transcription factors TcJAMYC1, TcJAMYC2, and TcJAMYC4. Front Plant Sci 6:115

Liu Y, Ji X, Nie X, Qu M, Zheng L, Tan Z, Wang Y (2015) Arabidopsis AtbHLH112 regulates the expression of genes involved in abiotic stress tolerance by binding to their E-box and GCG-box motifs. New Phytol 207:692–709

Maas C, Laufs J, Grant S et al (1991) The combination of a novel stimulatory element in the first exon of the maize Shrunken-1 gene with the following intron 1 enhances reporter gene expression up to 1000-fold. Plant Mol Biol 16:199–207

Maher L, Burton W, Salisbury P, Debonte L, Deng XM (2007) High oleic, low linolenic (HOLL) specialty canola development in Australia. The 12th International Rapeseed Congress, pp 22–24

Martínez-Rivas JM, Sperling P, Lühs W, Heinz E (2001) Spatial and temporal regulation of three different microsomal oleate desaturase genes (*FAD2*) from normal-type and high-oleic varieties of sunflower (*Helianthus annuus* L.). Mol Breeding 8:159–168

Mascarenhas D, Mettler IJ, Pierce DA, Lowe HW (1990) Intron-mediated enhancement of heterologous gene expression in maize. Plant Mol Biol 15:913–920

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T, Abe H, Yamaguchi-Shinozaki K (2003) Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. Plant J 34:137–148

Noir S, Bräutigam A, Colby T, Schmidt J, Panstruga R (2005) A reference map of the *Arabidopsis thaliana* mature pollen proteome. Biochem Biophys Res Commun 337:1257–1266

Park HC, Kim ML, Kang YH et al (2004) Pathogen-and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiol 135:2150–2161

Parra G, Bradnam K, Rose AB, Korf I (2011) Comparative and functional analysis of intron-mediated enhancement signals

reveals conserved features among plants. Nucleic Acids Res 39:5328–5337

Peng Q, Hu Y, Wei R, Zhang Y, Guan C, Ruan Y, Liu C (2010) Simultaneous silencing of *FAD2* and *FAE1* genes affects both oleic acid and erucic acid contents in *Brassica napus* seeds. Plant Cell Rep 29:317–325

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. Annu Rev Cell Dev Biol 28:489–521

Pirtle IL, Kongcharoensuntorn W, Nampaisansuk M, Knesek JE, Chapman KD, Pirtle RM (2001) Molecular cloning and functional expression of the gene for a cotton Δ-12 fatty acid desaturase (*FAD2*). BBA-Gene Struct Expr 1522:122–129

Rouster J, Leah R, Mundy J et al (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. Plant J 11:513–523

Scheffler JA, Sharpe AG, Schmidt H, Sperling P, Parkin IAP, Lühs W, Heinz E (1997) Desaturase multigene families of *Brassica napus* arose through genome duplication. Theor Appl Genet 94:583–591

Smooker AM, Wells R, Morgan C, Beaudoin F, Cho K, Fraser F, Bancroft I (2011) The identification and mapping of candidate genes and QTL involved in the fatty acid desaturation pathway in *Brassica napus*. Theor Appl Genet 122:1075–1090

Stålberg K, Ellerstöm M, Ezcurra I et al (1996) Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. Planta 199:515–519

Suh MC, Hahne G, Liu JR, Stewart CN Jr (2015) Plant lipid biology and biotechnology. Plant Cell Rep 34:517–518

Xiao G, Zhang HJ, Peng Q, Guan CY (2008) Screening and analysis of multiple copy of oleate desaturase gene (*fad2*) in *Brassica napus*. Acta Agronomica Sinica 34:1563–1568

Xiao G, Zhang ZQ, Yin CF, Liu RY, Wu XM, Tan TL, Guan CY (2014) Characterization of the promoter and 5′-UTR intron of oleic acid desaturase (*FAD2*) gene in *Brassica napus*. Gene 545:45–55

Yanagisawa S (2000) Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. Plant J 21:281–288

Yang YY, Yang SQ, Chen ZH, Guan CY, Chen SY, Liu ZS (2011) QTL analysis of 18-C unsaturated fatty acid contents in zero-erucic rapeseed (*Brassica napus* L.). Acta Agron Sin 37:1342–1350

Yang Q, Fan C, Guo Z, Qin J, Wu J, Li Q, Zhou Y (2012) Identification of *FAD2* and *FAD3* genes in *Brassica napus* genome and development of allele-specific markers for high oleic and low linolenic acid contents. Theor Appl Genet 125:715–729