Planta (2016) 244:467–478
DOI 10.1007/s00425-016-2519-1

ORIGINAL ARTICLE

Isolation and characterization of the *Jatropha curcas APETALA1* (*JcAP1*) promoter conferring preferential expression in inflorescence buds

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Received: 20 December 2015 / Accepted: 4 April 2016 / Published online: 19 April 2016

Abstract

Main conclusion **The 1.5 kb *JcAP1* promoter from the biofuel plant *Jatropha curcas* is predominantly active in the inflorescence buds of transgenic plants, in which the −1313/−1057 region is essential for maintaining the activity.**

Arabidopsis thaliana APETALA1 (*AP1*) is a MADS-domain transcription factor gene that functions primarily in flower development. We isolated a homolog of *AP1* from *Jatropha curcas* (designated *JcAP1*), which was shown to exhibit flower-specific expression in *Jatropha*. *JcAP1* is first expressed in inflorescence buds and continues to be primarily expressed in the sepals. We isolated a 1.5 kb *JcAP1* promoter and evaluated its activity in transgenic *Arabidopsis* and *Jatropha* using the *β*-glucuronidase (GUS) reporter gene. In transgenic *Arabidopsis* and *Jatropha*, the inflorescence buds exhibited notable GUS activity, whereas the sepals did not. Against expectations, the *JcAP1* promoter was active in the anthers of *Arabidopsis* and *Jatropha* and was highly expressed in *Jatropha* seeds. An analysis of promoter deletions in transgenic *Arabidopsis* revealed that deletion of the −1313/−1057 region resulted in loss of *JcAP1* promoter activity in the inflorescence buds and increased activity in the anthers. These results suggested that some regulatory sequences in the −1313/−1057 region are essential for maintaining promoter activity in inflorescence buds and can partly suppress activity in the anthers. Based on these findings, we hypothesized that other elements located upstream of the 1.5 kb *JcAP1* promoter may be required for flower-specific activation. The *JcAP1* promoter characterized in this study can be used to drive transgene expression in both the inflorescence buds and seeds of *Jatropha*.

Keywords Anther · *APETALA1* · Flower · Physic nut · Promoter · Seed

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Introduction

As a potential oilseed plant for renewable biodiesel production, *Jatropha curcas* (hereafter referred to as *Jatropha*), a member of the Euphorbiaceae family, has been studied for decades (Heller 1996; Sujatha et al. 2008; Divakara et al. 2010; de Argollo Marques et al. 2013). Considering the low genetic diversity of *Jatropha* (Tatikonda et al. 2009; Cai et al. 2010), transgenic breeding is a highly promising approach for directionally modifying characteristics of *Jatropha* in a short time, without the limitation of germplasm resources. To accelerate the process of the transgenic breeding of *Jatropha*, many efforts have been made to establish a genetic transformation system (Li et al. 2008; Joshi et al. 2010; Kumar et al. 2010; Pan et al. 2010) to analyze gene expression patterns (Chen et al. 2011; Sato et al. 2011; Li et al. 2015) and functions (Tang et al. 2011; Qu et al. 2012; Wei et al. 2012; Li et al. 2014). However, few studies have evaluated promoters in

Jatropha. The *CURCIN* promoter (*CP1*) and *curcin*-L promoter (*CP2*) isolated from *Jatropha* have been characterized in tobacco, in which *CP1* is specifically active in the seeds (Qin et al. 2009a), and *CP2* is a leaf-specific promoter induced by stresses (Qin et al. 2009b). The *JcNAC1* promoter is active in the roots, stems and leaves of seedlings, and *JcNAC1* promoter activity can be induced in guard cells by drought (Qin et al. 2014). The *JcSDP1* promoter is only active in *Jatropha* seeds, especially in the endosperm (Kim et al. 2014). The *JcMFT1* promoter is highly active in transgenic *Arabidopsis* seeds and can be induced by ABA in germinating seeds (Tao et al. 2014). The *JcUEP* promoter could serve as an alternative to the *CaMV35S* promoter for driving the constitutive overexpression of transgenes in *Jatropha* (Tao et al. 2015). It needs more efforts to characterize more promoters in *Jatropha*, which is one of the crucial regulation factors for efficient expression of transgenes in *Jatropha*.

In this study, we focused on isolating a reproductive tissue-specific promoter in *Jatropha* because the seed yield can be improved by modifying the flowering trait (Pan and Xu 2011; Chen et al. 2014; Pan et al. 2014). *APETALA1* (*AP1*) is a MADS-domain transcription factor gene that specifies floral meristem identity and functions as an A-class gene involved in floral organ formation (Mandel et al. 1992; Mena et al. 1995; Berbel et al. 2001; Litt and Kramer 2010; Chi et al. 2011). In *Arabidopsis*, *AP1* acts a hub that mediates the switch from floral initiation to flower formation (Kaufmann et al. 2010). Overexpression of *Arabidopsis AP1* in tomato and citrus results in early flowering and a reduction of the generation time in transgenic plants (Pena et al. 2001; Ellul et al. 2004). As expected, the *Arabidopsis AP1* promoter is active in floral primordia and young floral buds produced from primary and secondary inflorescences (Chou et al. 2001; Guan et al. 2002); thus, it has been used as a flower-specific promoter to analyze gene function in flowers or to modify flower traits. The *AP1* promoter directs the expression of the floral regulatory gene *SUPERMAN* (*SUP*) in *Arabidopsis* and tobacco, and the flowers of transgenic plants of both species exhibit fewer floral organs, consistent with an effect of *SUP* on cell proliferation (Yun et al. 2002). Expression of the cytokinin synthesis gene *IPT4* driven by the *AP1* promoter in *Arabidopsis* results in an increased flower number (Li et al. 2010). In the horticultural plant torenia, *AP1:MYB24-SRDX* transgenic plants produce open flowers with wavy petals and normal leaves, whereas *35S:MYB24-SRDX* transgenic plants exhibit unopened flower buds and glossy dark green leaves with curled margins (Sasaki et al. 2011). The use of flower-specific promoters has the advantage of defining modifications in flowers rather than other organs.

Here, we isolated the *Jatropha curcas APETALA1* (*JcAP1*) promoter and characterized its activity in *Arabidopsis* and *Jatropha*. In *Arabidopsis*, the *JcAP1* promoter was active only in inflorescence buds and anthers, and we found that the −1313/−1057 region was essential for promoter activity in inflorescence buds. However, in addition to the inflorescence buds, the *JcAP1* promoter was also highly active in *Jatropha* seeds.

Materials and methods

Plant materials

Jatropha curcas plants cultivated in Xishuangbanna, Yunnan Province, China, were used as previously described (Pan and Xu 2011). The *Arabidopsis thaliana* Col-0 ecotype, employed for transformation was grown at 22 °C with a 16 h light/8 h dark photoperiod.

qRT-PCR analysis in *Jatropha*

A cDNA sequence (GenBank accession no. KM610239) of the *APETALA1* (*AP1*) gene was identified from our *Jatropha* flower cDNA library (Chen et al. 2014). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the expression levels of *JcAP1* in various organs of adult *Jatropha* plants, including the roots, stems, leaves, inflorescence buds, female flowers, male flowers, pericarps and seeds at 42 days after pollination (DAP). Total RNA was isolated (Ding et al. 2008) and reverse transcribed using the PrimeScript® RT reagent kit with gDNA Eraser (TAKARA). qRT-PCR was performed with SYBR® Premix Ex Taq™ II (TAKARA) using the Roche 480 real-time PCR detection system (Roche Diagnostics). All of the gene expression data obtained via qRT-PCR were normalized to the expression of *JcGAPDH*. The primers used for qRT-PCR are listed in Table 1.

Cloning of the 5′ flanking region and determination of the transcription start site of *JcAP1*

The 5′ flanking region upstream of the translation start codon of *JcAP1* was isolated from *Jatropha* genomic DNA through genome walking (Siebert et al. 1995). For nested PCR, the *JcAP1* gene-specific primers GSP1 and GSP2 and the adaptor primers AP1 and AP2 were used. The *JcAP1* promoter was amplified via PCR and cloned into the pGEM-T Easy vector for sequencing. The putative *cis*-acting elements of the *JcAP1* promoter were analyzed using the PLACE database (Higo et al. 1999).

Table 1 Sequences of the primers used in this study

Name	Sequence (from 5′ to 3′)	Feature
AP1	GTAATACGACTCACTATAGGGC	Adaptor primer for genome walking
AP2	ACTATAGGGCACGCGTGGT	Adaptor primer for genome walking
GSP1	TCAACTGAACCCTACCTCTACCCATT	*JcAP1* gene-specific primer for genome walking
GSP2	ACAGCCAAAACCCAAGAAAATACCGA	*JcAP1* gene-specific primer for genome walking
XB989	CAATCAAAGCAACCTCAGCATCACACA	*JcAP1* gene-specific primer for 5′-RACE
XT95	GCTGCTAAGGCTGTTGGGAA	*JcGAPDH* gene primer for qRT-PCR
XT96	GACATAGCCCAATATTCCCTTCAG	*JcGAPDH* gene primer for qRT-PCR
XK714	GGGTTATTTTGAGGAAAGAAGAGGA	*JcAP1* gene primer for qRT-PCR
XK715	AAACAATCAAAGCAACCTCAGCATC	*JcAP1* gene primer for qRT-PCR
XT409	TGCTCTAGACTGTTACATATTACTATTA	For cloning the full-length promoter and construction of *JcAP1:GUS*, added *Xba*I site was underlined
XT412	CGCGGATCCTTCAACAAATATGTATAAAT	For cloning the full-length promoter and construction of *JcAP1:GUS*, added *Bam*HI site was underlined
XK93	TGCTCTAGATTCCGTAAAACCTTTCCAA	For construction of *D1:GUS*, added *Xba*I site was underlined
XK94	TGCTCTAGATTTTACGATGACGTGTAT	For construction of *D2:GUS*, added *Xba*I site was underlined
XK95	TGCTCTAGACATTGTATTTGGCACTAA	For construction of *D3:GUS*, added *Xba*I site was underlined
XK96	TGCTCTAGAAGTAAATGCTAAACGAAC	For construction of *D4:GUS*, added *Xba*I site was underlined
XK97	TGCTCTAGAAATCCTATTTATAACCCTT	For construction of *D5:GUS*, added *Xba*I site was underlined
XK314	GGATACCGAGGGGAATTTATGGAA	For TAIL-PCR amplifying the T-DNA right flanking sequence
XK315	TGACCTTAGGCGACTTTTGAACG	For TAIL-PCR amplifying the T-DNA right flanking sequence
XK316	CAGTTCCAAACGTAAAACGGCTTG	For TAIL-PCR amplifying the T-DNA right flanking sequence
AD1	ASCWGNTSAGNTSAGG	For TAIL-PCR amplifying the T-DNA right flanking sequence
AD2	TGNCASTCWGNANTCG	For TAIL-PCR amplifying the T-DNA right flanking sequence
AD3	GWANCTNASTCGNGTT	For TAIL-PCR amplifying the T-DNA right flanking sequence
AD4	TGNWCWGNTSANSACT	For TAIL-PCR amplifying the T-DNA right flanking sequence

To identify the transcription start site of *JcAP1*, rapid amplification of 5′-cDNA ends (5′-RACE) was performed with total RNA from *Jatropha* male flowers using the SMARTer® RACE 5′/3′ Kit (Clontech). The primers employed for genome walking and 5′-RACE are listed in Table 1.

Construction of *promoter-GUS* fusions

To generate the *JcAP1:GUS* plasmid, *Xba*I and *Bam*HI were used to digest pBI101 (Jefferson et al. 1987) and the pGEM-T Easy vector containing the 1.5 kb *JcAP1* promoter. The two fragments were linked using T4 ligase (Promega). The resulting construct, *JcAP1:GUS* (Fig. 3b), was transferred to *Agrobacterium tumefaciens* EHA105 and LBA4404 via electroporation (GenePulser Xcell, Bio-Rad). Strain EHA105 harboring the construct was employed to transform *Arabidopsis*; strain LBA4404 harboring the construct and pBI101 (promoterless, negative control, NC) was employed to transform *Jatropha*.

Plant transformation

Jatropha transformation was performed as described by Fu et al. (2015). After sterilization with 75 % (v/v) ethanol for 30 s, the mature seeds of *Jatropha* were sterilized with 10 % (v/v) sodium hypochlorite for 20 min. The embryos were removed from the seeds, and cotyledon explants were cut from the base of the cotyledons, leaving 3/4 of the papery cotyledons for co-cultivation with *Agrobacterium*. In co-cultivation experiments, 50 μM acetosyringone was added to the MS-Jc1 medium (Pan et al. 2010). After 2 days of co-cultivation, the explants were cultured in MS-Jc1 medium with 100 mg/L timentin for a 10-day recovery period and then subcultured in shoot-inducing medium (SIM, MS-Jc1 medium with 40 mg/L kanamycin and 100 mg/L timentin). After selection, the regenerated shoots were transferred to rooting medium [RM, half strength (1/2) MS medium with 0.2 mg/L IBA, 0.1 mg/L NAA and 100 mg/L timentin]. Finally, the putative *Jatropha* transformants were examined via TAIL-PCR, and positive

transgenic plants were cultivated in soil. *Arabidopsis* transformation was performed through the floral dip method (Clough and Bent 1998).

TAIL-PCR analysis

Thermal asymmetric interlaced PCR (TAIL-PCR) was performed with genomic DNA isolated from the leaves of the putative *Jatropha* transformants. The procedure was conducted as described by Liu et al. (1995). Three specific primers were designed based on the right border sequences of the T-DNA of pBI101. Four arbitrary degenerate (AD) primers (AD1, AD2, AD3 and AD4) and the T-DNA-specific primers XK314, XK315 and XK316 were employed to examine *JcAP1:GUS* transformants. The primers used for these assays are listed in Table 1. After the tertiary reaction, the TAIL-PCR products were sequenced and analyzed using the Jatropha Genome Database (http://www.kazusa.or.jp/jatropha/).

Histochemical and fluorometric GUS assay

For histochemical GUS staining, various tissues of transgenic *Jatropha* and *Arabidopsis* were incubated in GUS assay buffer with 50 mM sodium phosphate (pH 7.0), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 0.5 % Triton X-100 and 1 mM X-Gluc at 37 °C overnight, then cleared in 70 % ethanol (Jefferson et al. 1987). The samples were examined via stereomicroscopy (Leica M80).

To examine the activity of the *JcAP1* promoter in different tissues, a fluorometric GUS assay was performed following the protocol described by Jefferson et al. (1987), which was modified by adding 2 mM MUG to the reaction buffer. Fluorescence was examined with a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation). The protein concentrations of the plant extracts were measured using the Bradford method (1976).

Results

The expression pattern of *JcAP1* in *Jatropha*

We identified a cDNA of *AP1* (GenBank accession no. KM610239) from our *Jatropha* flower cDNA library (Chen et al. 2014). *JcAP1* encodes a MADS-box transcription factor that contains two conserved regions: a MADS-domain and a K domain (Fig. 1a). JcAP1 is highly similar to AP1 homologs from other plant species, and it is most closely related to PtAP1 from *Populus trichocarpa* (Fig. 1b).

To explore the expression pattern of *JcAP1* in *Jatropha*, qRT-PCR was performed with total RNAs extracted from various tissues. The results indicated that *JcAP1* is a flower-specific gene that is predominantly expressed in floral tissues, especially in inflorescence buds (Fig. 2). Because most *AP1* genes are involved in the development of floral organs, we also examined *JcAP1* expression in each organ of male and female *Jatropha* flowers. As shown in Fig. 2, *JcAP1* was highly expressed in the sepals, while the levels in the petals and stamens were low in male flowers; in female flowers, the highest level was also found in the sepals, which was approximately two times that in the petals, and the expression level in the pistils was very low. The expression pattern of *JcAP1* in floral organs was consistent with that of A-class genes in other plants (Coen and Meyerowitz 1991; Litt and Kramer 2010).

Isolation and sequence analysis of the *JcAP1* promoter

On the basis of expression data, a 1.5 kb *JcAP1* promoter fragment (−1313/+150) (GenBank accession no. KM610240) was isolated from *Jatropha* by genome walking (Siebert et al. 1995). The transcription start site, determined by 5′-RACE, was located 150 nucleotides upstream of the translation start codon of *JcAP1* (Fig. 3a). The putative *cis*-acting elements were analyzed using the PLACE database (Higo et al. 1999). The *JcAP1* promoter sequence and putative plant regulatory elements are shown in Fig. 3a. The analysis revealed that the *JcAP1* promoter contains a CArG box, which acts as a binding site for MADS-box proteins and is an important element mediating the regulatory effect on MADS-box genes (Tilly et al. 1998). Another important element of the promoter is a binding site (CCAATGT) for the LEAFY protein, which is a transcriptional factor that activates *AP1* expression in *Arabidopsis* (Wagner et al. 1999). This promoter also contained some pollen-specific elements, including the POLLEN1LELAT52 motif and the GTGANTG10 motif, which are essential for the pollen-specific expression of tomato *LAT52* and tobacco *g10*, respectively (Twell et al. 1991; Muschietti et al. 1994; Rogers et al. 2001).

Characterization of *JcAP1* promoter activity in transgenic *Arabidopsis*

To test the activity of the promoter, *JcAP1:GUS* was transformed into *Arabidopsis* for preliminary analysis. GUS staining was examined in the T2 generation of five independent transgenic lines. The results showed that GUS activity was first detectable in the inflorescence buds and then in the anthers where pollen staining was intense (Fig. 4). The staining results in transgenic *Arabidopsis* indicated that the activity of the *JcAP1* promoter was confined to flowers; however, the observed activity was not consistent with *JcAP1* expression in *Jatropha*, which was abundant in the sepals rather than the stamens (Fig. 2).

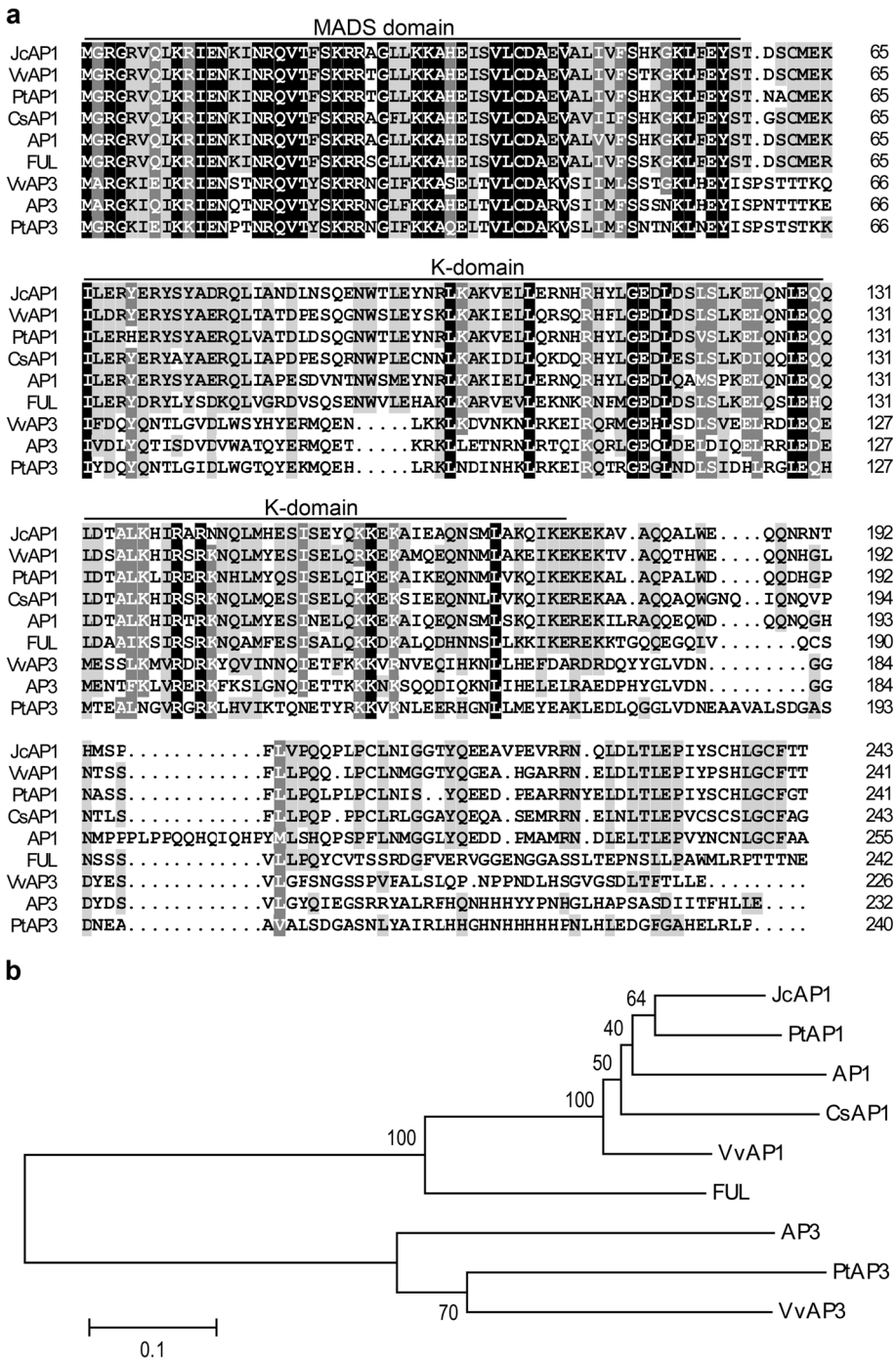

Fig. 1 A comparison of JcAP1 and its homologs. **a** Alignment of the deduced amino acid sequence of JcAP1 (accession No. KM610239) with that of *Arabidopsis thaliana* AP1 (accession No. CAA78909), FUL (accession No. NP_568929) and AP3 (accession No. BAA04665); *Vitis vinifera* VvAP1 (accession No. NP_001268210) and VvAP3 (accession No. ABN71371); *Citrus sinensis* CsAP1 (accession No. NP_001275828); *Populus trichocarpa* PtAP1 (accession No. XP_002311353); and *Populus tomentosa* PtAP3 (accession No. AAQ83493). Identically and partially conserved amino acid sequences are shown in *black* and *gray*, respectively. The conserved regions, MADS-domain and K domain of JcAP1 are indicated with overlining. **b** Phylogenetic analysis of JcAP1 and its homologs. The tree was constructed using MEGA 5.0 software and the neighbor-joining (N-J) method. The unrooted N-J dendrogram was generated from an alignment of the deduced amino acids with the ClustalW program. One thousand replicates were used for the bootstrap analysis. The *scale bar* indicates the average number of substitutions per site

This result led us to hypothesize that the pollen-specific elements present in the *JcAP1* promoter region may be hyperactive in the heterogeneous expression system.

Deletion analysis of the *JcAP1* promoter in transgenic *Arabidopsis*

A series of 5′ deletions of the *JcAP1* promoter (Fig. 5a) were generated to analyze the regulatory effect of different regions of the promoter. After a histochemical GUS assay, we found that compared with the full-length promoter, GUS activity could not be detected in the inflorescence buds of all five deletions (D1–D5), whereas staining was still observed in the anthers, except in the D5 (Fig. 5b). The disappearance of GUS activity in the inflorescence buds of plants transformed with the D1 construct indicated that the −1313/−1057 region is essential for inflorescence bud specificity.

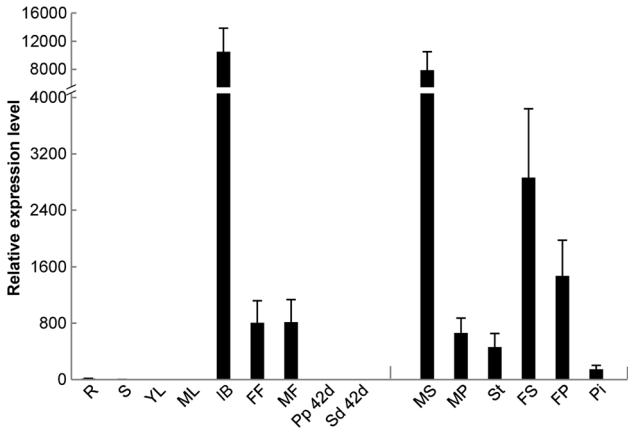

Fig. 2 Expression pattern of *JcAP1* in *Jatropha*. Samples from adult plants: roots (*R*), stems (*S*), young leaves (*YL*), mature leaves (*ML*), inflorescence buds (*IB*), female flowers (*FF*), male flowers (*MF*), pericarps at 42 DAP (Pp 42 d), seeds at 42 DAP (Sd 42 d), male sepals (*MS*), male petals (*MP*), stamens (*St*), female sepals (*FS*), female petals (*FP*) and pistils (*Pi*). Equivalent qRT-PCR results were obtained from duplicate biological replicates. The *error bars* denote the SD from three technical replicates. The values were normalized to the expression of the reference gene *JcGAPDH* (Zhang et al. 2013)

To further investigate the regions of the *JcAP1* promoter involved in the regulation of anther-specific activity, we detected GUS expression in the anthers of plants transformed with constructs containing the full-length sequence and deletions of the *JcAP1* promoter. As shown in Fig. 5a, removing the −1313/−1057 region resulted in an increase in GUS expression for all of the deletions except for the shortest (D5), and D1 exhibited the highest expression level. This result indicated that the −1313/−1057region represses the activity of the *JcAP1* promoter in anthers. Deletion of the −1057/−796 region from D1 to produce D2 led to a reduction of the GUS expression level, while further deletion to position −521 (D3) resulted in almost the same expression level observed for D2. Removal of the −521/−242 fragment from D3 to produce D4 caused a further decrease in the GUS expression level. Consistent with the GUS staining results, a fluorometric GUS assay revealed very low GUS activity for D5 (Fig. 5a, right panel). These results suggested that the −1057/−796 and −521/+19 regions are capable of inducing *JcAP1* promoter activity in anthers, while the −796/−521 region shows no significant effects.

Characterization of *JcAP1* promoter activity in transgenic *Jatropha*

Following evaluation in *Arabidopsis*, the *JcAP1:GUS* construct was transformed into *Jatropha* to characterize the activity of the *JcAP1* promoter. Transformed plantlets were generated via kanamycin selection and validated through PCR amplification. Then, the PCR-positive transformants were examined via TAIL-PCR to confirm stable transgene integration. The T-DNA right border insert from pBI101 was used for integration analysis. The sequences of the T-DNA integration sites (i.e., the T-DNA/*Jatropha* genomic DNA junction regions) obtained from four transformant events (B1, B5, B12, and B14) selected randomly are listed below the sequence of the T-DNA right border of pBI101 in Table 2. The results showed that either the entire T-DNA right border sequence (B1 and B5) or part of it (B12 and B14) had been lost and that the transgenes were integrated into the genome of *Jatropha* transformants. In addition, the integration sites in the *Jatropha* genome were different between different insertion events, indicating that the plants were independent lines for the transformation event. The transgenic plantlets were grown in soil for further analysis.

A histochemical GUS assay was first performed on regenerated transgenic shoots, and no GUS staining was observed (data not shown). After the transgenic plantlets had grown into the adult plants, various tissues, including roots, stems, young and mature leaves, shoot apices, inflorescence buds, female and male flowers, fruits at 12 DAP and seeds at 25 DAP, were collected for histochemical GUS assay. The results (Fig. 6) showed that GUS staining was strong in the shoot apices, inflorescence buds, male flowers and seeds; weak in the stems and female flowers, and absent from the roots, leaves and pericarps. No GUS staining was detected in the negative control. In male flowers, the anther was also stained (Fig. 6), which is consistent with the results from transgenic *Arabidopsis* (Fig. 4c). Thus, the activity of the *JcAP1* promoter in anthers was not caused by the previously described heterogeneous expression. This result demonstrated that the *JcAP1* promoter isolated here is active in anthers. Furthermore, the *JcAP1* promoter is active in shoot apices and seeds, differing from the expectation of expression restricted to inflorescence buds and flowers.

Next, we compared *JcAP1* promoter activity in different tissues of adult *Jatropha* plants via a fluorometric GUS assay. Tissues from five independent lines of the T0 generation were used for detection, including the roots, stems, young and mature leaves, shoot apices, inflorescence buds, female and male flowers, fruits at 12 DAP, pericarps, and seeds at 25 DAP. As shown in Fig. 7, the highest GUS expression level was detected in inflorescence buds, followed by the seeds at 25 DAP. The expression levels in shoot apices and male flowers were approximately equal but were less than half of the levels observed in seeds at 25 DAP. Although all four tissues exhibited clearly visible GUS staining, activity differed from the tissues. Consistent with the GUS staining results, fluorometric GUS activity in the stems, female flowers and fruits (12 DAP) was lower than in the shoot apices and male flowers. These results

Fig. 3 *JcAP1* promoter sequence and promoter reporter gene construct. **a** Nucleotide sequence of the *JcAP1* promoter. The transcription start site (+1) is in *bold* and *italic*. The start codon ATG is in *bold* and *boxed*. Putative regulatory elements on both strands are shown in *bold* and *underlined*. **b** Schematic representation of the T-DNA regions of the *JcAP1:GUS* binary vector used for transformation

a

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-1313   CTGTTACATA TTACTATTAC GTTTTCCATA ATCTGCAAGT TCTATCAACA ACAAAACACA
-1253   AAAATAACTA AATATATTAA ATATCGAAAG GCATAGTTTT TATGGGTTTG CCTTTTCTTT
                                                  SEF4 motif
-1193   TAATTTGATA TGTACAAATT CAACTTTTTT TTGAAAAAAA AAGAAGTACA AATTCACCTA
                                    CArG                DOF core
-1133   TGAGGAACCG ATTGCATAAT ATGGACAGTA GATGGCCTTT TTTTAATGTT GTTTTTATAC
                                                                SEF4 motif
-1073   TACGACCAAC GTAGTATTCC GTAAAACCTT TCCAATAGTT ACAATTTCAG ATTTTGAATC
-1013   TTTTAACCTT TTTAGTAGTA TTCAGTTTTT ACACCAGCTT AATCATGATT TATCTTGAAT
-953    TGTTTATATC CATTTTTGCT TTAGAGCAAT TCTAATGTGA CCGTAGGTTG TGGCTTATAT
                  SEF4 motif                    GTGANTG10
-893    AGCATATTAA ATAGAAGATA TTAATAATGA TGTGACTATA AGTCTATTTG TAAATATTAA
                           TATA box       GTGANTG10
-833    ATTTGAAAAC GGGCTCTCCA ATGTATCATA ATAGGATTTT TACGATGACG TGTATTGTTT
                        LEAFYATAG              SEF4 motif
-773    TTTTAAAATT ATATCTAATT ATAATAAAAG AAAATAAGTT GAAATTTGAA AAATATTCTG
                                    POLLEN1LELAT52
-713    TTGAAATTTG AGACCTCTAA TTTCTCGTAT AAGTGAAAAG ACTAATCAAA CCACGGTTGA
                                          GTGANTG10
-653    TATATATTAC ATTAAATTTT TTTTATTTAT GCATTATAAA TCATTATAGA AGTGAAAAGA
                                 TATA box      TATA box          GTGANTG10
-593    ATAACCAGAT TAGACACTTG ATTTAAGATT ATTTTAGAAT TTTTTACTTG TGGATGGTAA
                           E box          TATA box
-533    GTCATTATAA GTCATTGTAT TTGGCACTAA ACAGTTGCAT AATAATACCA ATTCAGAATT
                                          E box
-473    AAGAGTTAAT TACTAAGAAA AAAAAAATCA TAGTTTGGTT AAAGAAACAA AAAACTCCGC
                        POLLEN1LELAT52              POLLEN1LELAT52
-413    CGTCATACTA ATAATGAAAG TATGTGTTTG TTTGGGTGGA CGACAGGGTT CGACCAATAC
                           DOF core
-353    CAAGGAGGTT TTTCAATAGT CCCGTAGAGG GATCTGACAC TGCCATGCCA CCATTAGTCT
-293    CTGTGGCCTC GAATATTACA TTCCTAAATA AAAATTAGTA GTATAAAAGA AAGTAAATGC
                                                POLLEN1LELAT52
-233    TAAACGAACA TTAAATAAGA GTTCTTTATA CAAGAAAATG AGGTTTTTTG CACATCAGGT
                                       POLLEN1LELAT52
-173    AATACGGACT CTGTAGTACA AGCAATAGCC AATGAGAAAT CGACAGCAAA TTCAAAAAAA
                                       POLLEN1LELAT52
-113    AAAAACCACT CTGGTATGTG AAAACCTGCC CTTTTCCATA AAAACCACTA TCCCCCTCTC
                           GTGANTG10                                  +1
-53     TACCTCTCCA TTTTCCCATT CTATATATGT GCATGCTTTC TTGGGAAAGC TTCATATGTA
                                                        DOF core
+8      GCAAGAAGAA AAATCCTATT TATAACCCTT TCTCTATTTA TACAGCCTCT CTTTTGGTTT
+68     TTCTTTTTCG GTATTTTCTT GGGTTTTGGC TGTTTGGGTT ATTTTGAGGA AAGAAGAGGA
+128    AAAATTTATA CATATTTGTT GAAATG
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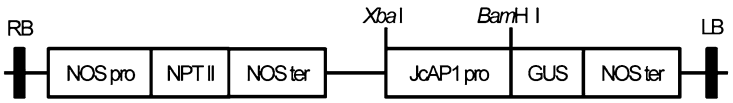
b



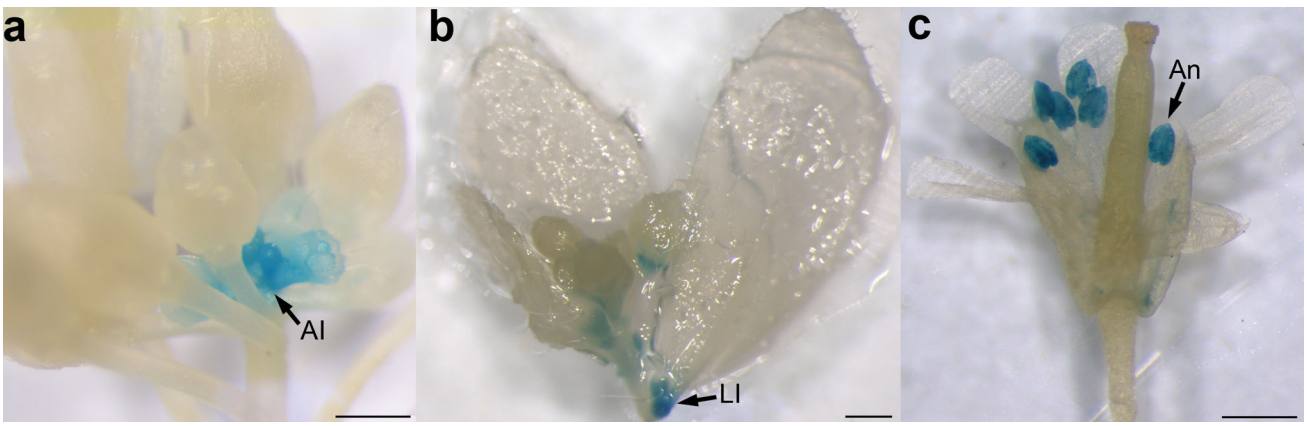


Fig. 4 Histochemical GUS staining of transgenic *Arabidopsis* harboring the *JcAP1:GUS* fusion. **a**, **b** Inflorescence buds, **c** flowers. *AI* apical inflorescence buds, *LI* lateral inflorescence buds, *An* anthers. All *bars* are 0.5 mm

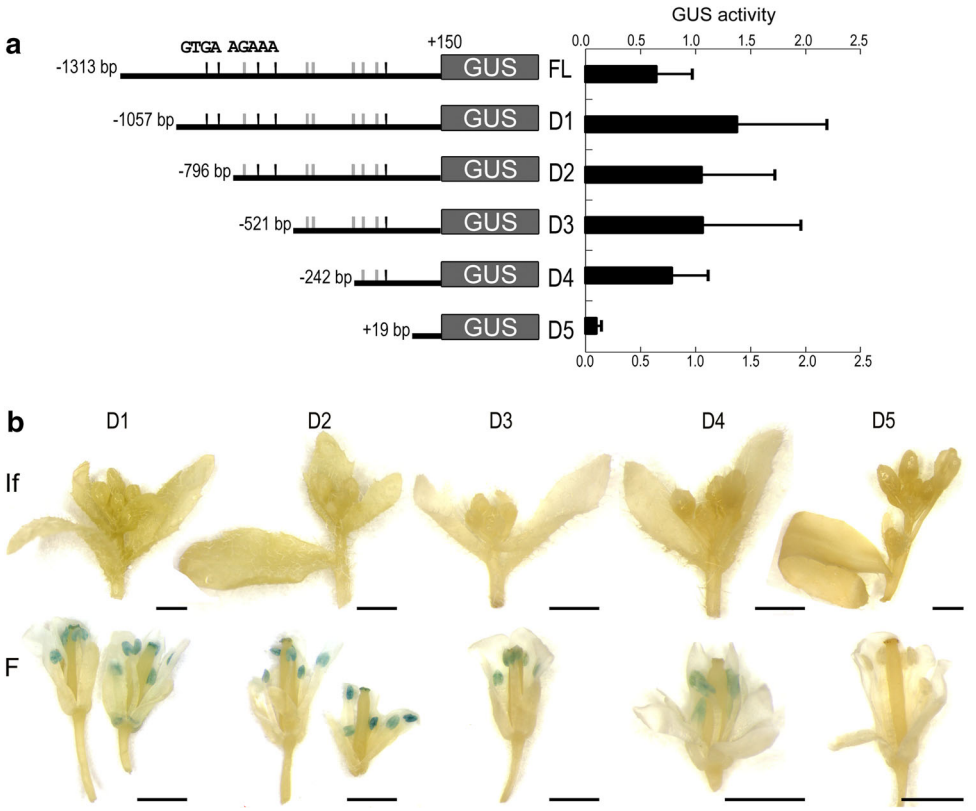


Fig. 5 Deletion analysis of the *JcAP1* promoter. **a** Schematic representation of different *JcAP1* promoter deletions (*left panel*) and the fluorometric assay of GUS activity in the anthers (*right panel*). *FL* full-length *JcAP1* promoter, *D1–D5* five deletions. GTGA: GTGANTG10 motif (*black vertical bars*); AGAAA: POLLEN1LELAT52 motif (*gray vertical bars*). The presented GUS activity values are the averages from five independent transgenic lines from the T2 generation, and the *error bars* denote the SD. The GUS activities are expressed as nmol of 4-MU produced per min per mg of protein and were measured three times. **b** Histochemical GUS staining of transgenic *Arabidopsis* harboring deletions. *If* inflorescences, *F* flowers. All *bars* are 1 mm

Table 2 Sequence analysis of the T-DNA right border (underlined) and *Jatropha* genomic DNA (bold)

Plasmid	No.*	Right boundary T-DNA/ plant genomic DNA junctions
pBI101		--CAGTTTAAACTATCAGTGTTTGACAGGATATATTGGCGGGTAAAC--
pJcAP1:GUS	B1	--TCGTTTCCCGCCTTCAGTTTAAA**GAAGAGCCGGACTGAAACCGGCTAACTGCCA**--
	B5	--TGGCTCCTTCAACGTTGCGGTTC**CCTTACCACTCGACTCATCGCAAGAGAAAAAT**--
	B12	--CAGTTTAAACTATCAGTGTTTGA**GTATGTATCTTCCTTTACACTTCCTAGTTCCTA**--
	B14	--CAGTTTAAACTATCAGTGTTTGA**TCTTTATGTACGTAAAGTCATTAAGCATTCCTTT**--

* Serial number of transgenic *Jatropha* plants transformed with *JcAP1:GUS*

indicated that the *JcAP1* promoter is primarily active in inflorescence buds and seeds (25 DAP).

Discussion

AP1 is a MADS-box transcription factor that plays an important role in the initiation of flowering and floral organ formation (Kotoda et al. 2002; Murai et al. 2003; Huang et al. 2014). *AP1* is first expressed in floral meristems and continues to be expressed in the outer whorls of the flower. In maize, *ZAP1* expression is restricted to the inflorescences and non-reproductive organs of male and female flowers (Mena et al. 1995). Similar to *ZAP1*, a high expression level of *JcAP1* was first detected in inflorescence buds and then in male and female sepals (Fig. 2). However, the expressions of other *AP1* orthologs exhibit different patterns. *CsAP1* from *Citrus sinensis* is expressed not only in the outer whorls of the sepal and petal, but also in the inner whorls of the stamen and carpel (Pillitteri et al. 2004). A hybrid aspen *LAP1* was found to be expressed at a high level in the apex and may function mainly in seasonal growth rather than flowering (Azeez et al. 2014). In this study, because *JcAP1* was shown to be flower-specific, we isolated its 5′-flanking region as a tissue-specific promoter and characterized this region in transgenic *Arabidopsis* and *Jatropha*.

The *JcAP1* promoter showed marked activity in inflorescence buds in both transgenic *Arabidopsis* (Fig. 4) and *Jatropha* (Fig. 7), which was consistent with the *JcAP1* expression pattern in *Jatropha* (Fig. 2). Through deletion analysis, we found that the −1313/−1057 region is

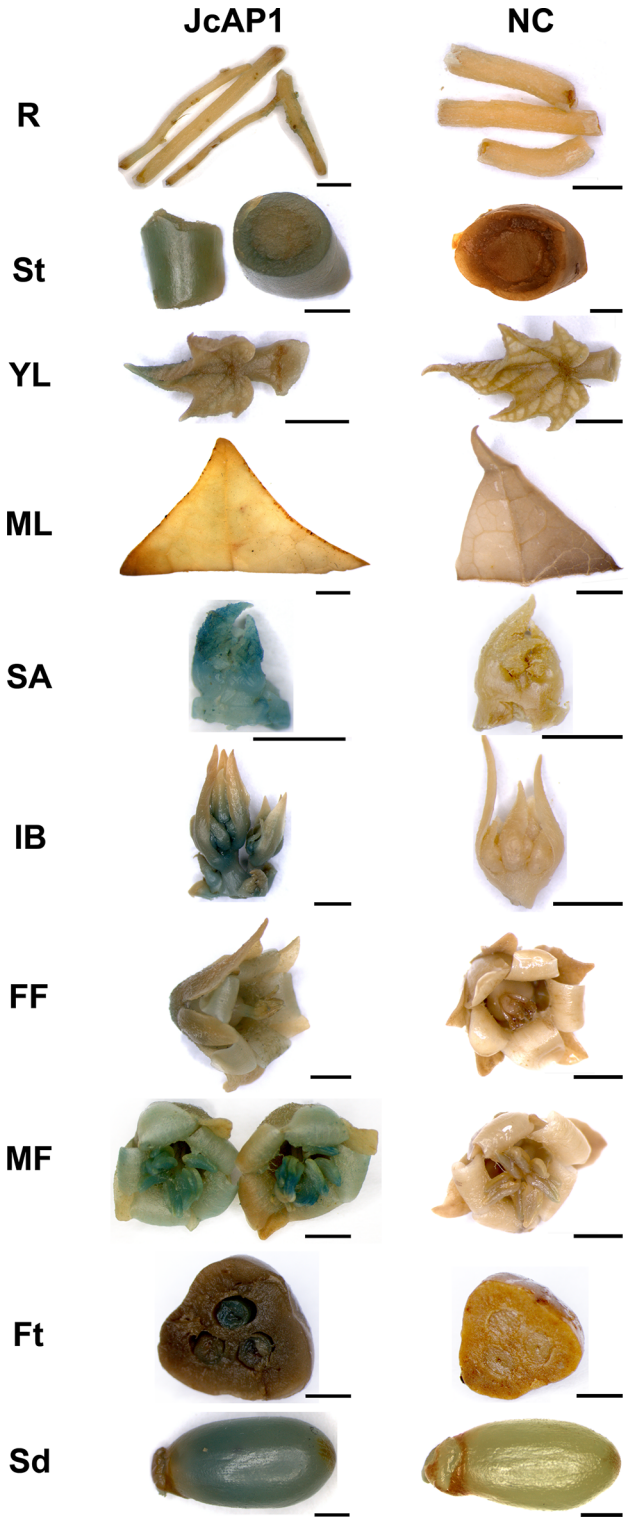


Fig. 6 Histochemical GUS staining in various tissues of adult transgenic *Jatropha* plants (T0) harboring *JcAP1:GUS* and pBI101 (*NC* non-transgenic control). *R* roots, *St* stems, *YL* young leaves, *ML* mature leaves, *SA* shoot apices, *IB* inflorescence buds, *FF* female flowers, *MF* male flowers, *Ft* fruits at 12 DAP, *Sd* seeds at 25 DAP. All *bars* are 2 mm

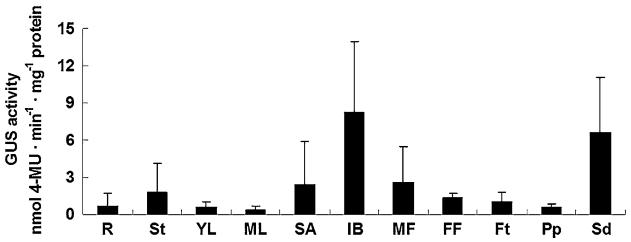


Fig. 7 Fluorometric assay of GUS activity in adult transgenic *Jatropha* plants (T0). *R* roots, *St* stems, *YL* young leaves, *ML* mature leaves, *SA* shoot apices, *IB* inflorescence buds, *MF* male flowers, *FF* female flowers, *Ft* fruits at 12 DAP, *Pp* pericarps at 25 DAP, *Sd* seeds at 25 DAP. The presented values are the averages from five independent transgenic lines, and the *error bars* denote the SD. GUS activities were measured three times

essential for *JcAP1* promoter activity in inflorescence buds. In addition, we identified a CArG box in this region, which is an important element mediating the regulatory effect of MADS-box proteins. In *Arabidopsis*, *APETALA3* (*AP3*) is a MADS-box gene that functions in the control of petal and stamen development (Kramer et al. 1998). Three CArG boxes in the *AP3* promoter mediate both positive and negative effects on the establishment and maintenance of the *AP3* expression pattern (Tilly et al. 1998). Therefore, the absence of the CArG box in deletion constructs of the *JcAP1* promoter (D1–D5, Fig. 5a) may completely abolish inflorescence bud activity. In floral organs, *JcAP1* was expressed highly in sepals but was expressed at low levels in stamens. However, GUS staining was not observed in either *Arabidopsis* (Fig. 4c) or *Jatropha* sepals (Fig. 6); on the contrary, the anthers were stained, and *Arabidopsis* pollen showed intense staining (Fig. 4c). We also noted that the *JcAP1* promoter contained some pollen-specific elements, including six POLLEN1LELAT52 motifs and five GTGANTG10 motifs, and promoter activity in the anthers decreased with the step-down numbers of these elements. In particular, deleting the −1057/−796 region, containing two GTGANTG10 motifs, and the −521/+19 region, containing five POLLEN1LELAT52 motifs, caused considerable loss of activity in the anthers (Fig. 5). Thus, we assumed that these elements confer the pollen-specific activity of the *JcAP1* promoter. Moreover, when the −1313/−1057 region was removed, *JcAP1* promoter activity in the anthers increased until the pollen-specific elements had been completely deleted; suggesting that the CArG box in the −1313/−1057 region repressed the pollen-specific elements. Similarly, the third CArG box (CArG3) in the *Arabidopsis AP3* promoter is required to maintain a low level of gene expression during early floral stages, and the GUS expression level was found to increase when CArG3 was mutated (Tilly et al. 1998).

In addition, the *JcAP1* promoter was unexpectedly active in regions beyond the flowers, showing a high expression level in the seeds of transgenic *Jatropha*. This result is consistent with seed-specific elements being present in this promoter, such as the E box (Kawagoe and Murai 1992; Stålberg et al. 1996), the DOF core sequence (Yanagisawa and Schmidt 1999), and the SEF motif (Lessard et al. 1991). The *JcAP1* expression, however, was not detected in *Jatropha* seeds (Fig. 2). Similarly, the 920 bp potato *ubi3* promoter was shown to be insufficient to achieve wound- and ethylene-dependent activation although native *ubi3* expression in tubes is induced by wounding and ethylene treatments (Garbarino and Belknap 1994). Moreover, the gene internal sequences may also take part in the regulation of gene expression. The parsley *4CL-1* gene expression was developmentally regulated by light and stresses, such as pathogen infection, UV-irradiation and wounding. But the *4CL-1* promoter alone was only sufficient to direct gene cell-specific expression. Actually, the exonic sequences were required, in addition to the promoter, for the *4CL-1* gene expression induced by fungal elicitor or light treatment (Douglas et al. 1991). The *Arabidopsis ACT2* promoter could not confer the strong expression of *ACT2* throughout the vegetative tissues unless it was associated with the first intron (Jeong et al. 2009). Hence, because an isolated promoter may not reveal the full expression pattern of a gene, it is necessary to evaluate promoter activity in target plant species.

Taken together, our findings indicate that the cooperation of multiple *cis*-acting elements in the *JcAP1* promoter is required to confer accurate activity in transgenic plants. Although the 1.5 kb *JcAP1* promoter did not exhibit the same expression pattern in transgenic *Arabidopsis* and *Jatropha* as was observed for *JcAP1* mRNA in *Jatropha* plants, this region could be used as a reproductive tissue-specific promoter in transgenic studies of *Jatropha*.

Author contribution statement Y-B Tao and Z-F Xu designed research and wrote the paper, Y-B Tao performed research, L-L He helped in data analysis, and L-J Niu helped in collection of plant materials.

Acknowledgments This work was supported by funding from the National Natural Science Foundation of China (31370595), the CAS 135 program (XTBG-T02), and the Top Science and Technology Talents Scheme of Yunnan Province (2009CI123). The authors gratefully acknowledge the Central Laboratory of the Xishuangbanna Tropical Botanical Garden for providing research facilities.

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