Ectopic expression of Jatropha curcas APETALA1 *(JcAP1*) caused early flowering in Arabidopsis, but not in Jatropha

Mingyong Tang, Yan-Bin Tao, Zeng-Fu Xu

Jatropha curcas is a promising feedstock for biofuel production because Jatropha oil is highly suitable for the production of biodiesel and bio-jet fuels. However, Jatropha exhibits a low seed yield as a result of unreliable and poor flowering. *APETALA1* (*AP1*) is a floral meristem and organ identity gene in higher plants. The flower meristem identity genes of Jatropha have not yet been identified or characterized. To better understand the genetic control of flowering in Jatropha, an *AP1* homolog (*JcAP1*) was isolated from Jatropha. An amino acid sequence analysis of JcAP1 revealed a high similarity to the AP1 proteins of other perennial plants. *JcAP1* was expressed in inflorescence buds, flower buds, sepals and petals. The highest expression level was observed during the early developmental stage of the flower buds. The overexpression of *JcAP1* using the cauliflower mosaic virus (CaMV) 35S promoter resulted in extremely early flowering and abnormal flowers in transgenic Arabidopsis plants. Several flowering genes downstream of *AP1* were up-regulated in the *JcAP1*-overexpressing transgenic plant lines. Furthermore, *JcAP1* overexpression rescued the phenotype caused by the Arabidopsis AP1 loss-of-function mutant *ap1-11*. Therefore, *JcAP1* is an ortholog of *AtAP1,* which plays a similar role in the regulation of flowering in Arabidopsis. However, the overexpression of *JcAP1* in Jatropha using the same promoter resulted in little variation in the flowering time and floral organs, indicating that *JcAP1* may be insufficient to regulate flowering by itself in Jatropha. This study helps to elucidate the function of *JcAP1* and contributes to the understanding of the molecular mechanisms of flower development in Jatropha.

Ectopic expression of *Jatropha curcas APETALA1* (*JcAP1*) caused early flowering in Arabidopsis, but not in Jatropha

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18

19 # Abstract

20 *Jatropha curcas* is a promising feedstock for biofuel production because Jatropha oil is highly
21 suitable for the production of biodiesel and bio-jet fuels. However, Jatropha exhibits a low seed
22 yield as a result of unreliable and poor flowering. *APETALA1* (*AP1*) is a floral meristem and organ
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24 identified or characterized. To better understand the genetic control of flowering in Jatropha, an
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26 revealed a high similarity to the AP1 proteins of other perennial plants. *JcAP1* was expressed in
27 inflorescence buds, flower buds, sepals and petals. The highest expression level was observed
28 during the early developmental stage of the flower buds. The overexpression of *JcAP1* using the
29 cauliflower mosaic virus (CaMV) 35S promoter resulted in extremely early flowering and
30 abnormal flowers in transgenic Arabidopsis plants. Several flowering genes downstream of *AP1*
31 were up-regulated in the *JcAP1*-overexpressing transgenic plant lines. Furthermore, *JcAP1*
32 overexpression rescued the phenotype caused by the Arabidopsis *AP1* loss-of-function mutant
33 *ap1-11*. Therefore, *JcAP1* is an ortholog of *AtAP1*, which plays a similar role in the regulation of
34 flowering in Arabidopsis. However, the overexpression of *JcAP1* in Jatropha using the same
35 promoter resulted in little variation in the flowering time and floral organs, indicating that *JcAP1*
36 may be insufficient to regulate flowering by itself in Jatropha. This study helps to elucidate the
37 function of *JcAP1* and contributes to the understanding of the molecular mechanisms of flower
38 development in Jatropha.

39 **Keywords:** Jatropha, physic nut, Arabidopsis, *APETALA1*, flower identity, flowering

40

41 Introduction

42 With the decreasing availability of fossil fuels and the deteriorating trend of environmental
43 pollution, biodiesel has garnered significant attention as an alternative fuel (Mofijur et al. 2016).
44 Physic nut (*Jatropha curcas* L.) is a perennial plant that belongs to the *Euphorbiaceae* family.
45 Jatropha is monoecious, with male and female flowers borne on the same inflorescence (Divakara
46 et al. 2010; Pandey et al. 2012; Wu et al. 2011). The potential benefit of growing Jatropha as a
47 cash crop for biofuel in tropical and sub-tropical countries is now widely recognized (Akashi 2012;
48 Khalil et al. 2013; Pua et al. 2011). Jatropha has been propagated as a unique plant with biodiesel
49 potential because of its multipurpose value, high oil content, high biomass productivity,
50 adaptability to marginal land under a variety of agro-climatic conditions, and non-competitiveness
51 with food production (Akashi 2012; Khalil et al. 2013; Pandey et al. 2012; Pua et al. 2011). The
52 oil content of Jatropha seeds and kernels ranges from 30-40% and 40-50% by weight, respectively
53 (Pan & Xu 2011; Sinha et al. 2015). Oil from Jatropha contains high levels of polyunsaturated
54 fatty acids; therefore, Jatropha is suitable as a feedstock for the production of biodiesel and bio-jet
55 fuel (Ong et al. 2011; Pramanik 2003). The whole-genome sequence and genetic mapping of
56 Jatropha have been reported (Hirakawa et al. 2012; Wu et al. 2015), and several genetic
57 transformation methods have been established (Fu et al. 2015; Gu et al. 2015; Kajikawa et al. 2012;
58 Kumar et al. 2010; Mao et al. 2013; Misra et al. 2012; Pan et al. 2010). Consequently, it is
59 convenient to clone Jatropha genes and analyze their functions. However, the potential of Jatropha

as a biofuel plant is limited by its low seed production (King et al. 2015). Despite the clear evidence of the abundant biomass generated by Jatropha, these data are not indicative of high seed productivity (Ghosh et al. 2007). Jatropha exhibits an overabundance of vegetative shoots and leaves in that could develop into reproductive shoots under suitable conditions. Thus, a reduction of undesired vegetative growth is imperative (Ghosh et al. 2010; Tjeuw et al. 2015). In addition, unreliable and poor flowering is an important factor that contributes to low seed productivity in Jatropha (Divakara et al. 2010). Therefore the elucidation of the genetic basis of flowering in Jatropha would be helpful for the molecular breeding of high-yielding Jatropha cultivars.

The *APETALA1* (*AP1*) was identified as a floral meristem identity gene to regulate flowering in many plant species. Mandel et al. (1992) reported that *AP1* encodes a putative transcription factor containing a MADS domain. This gene acts locally to specify the identity of the floral meristem and determine sepal and petal development. *AP1* and *LEAFY* (*LFY*) are pivotal in the switch to the reproductive phase. During floral initiation, a positive feedback loop between *AP1* and *LFY* is mediated by direct interactions (Kaufmann et al. 2010; Liljegren et al. 1999; William et al. 2004). After transition to flowering, the expression of *AP1* appears to be only indirectly affected by *LFY* (Wagner et al. 1999).. The *AP1*, *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) genes act redundantly to control the flower meristem identity and inflorescence architecture by affecting *LFY* and *TFL1* expression levels (Ferrándiz et al. 2000). *AP1* and *CAL* are expressed in floral meristems and developing sepal and petal primordial cells (Blazquez et al. 2006; Mandel et al. 1992).

In Arabidopsis *ap1* mutants, the sepals are converted to bract-like structures, the petals are absent, the bract-like organs of the first whorl subtend secondary flowers in the second whorl, and tertiary flowers can also form (Bowman et al. 1993; Irish & Sussex 1990; Mandel et al. 1992; Ng & Yanofsky 2001). Recent studies have indicated that *AP1* can regulate cytokine levels through the suppression of cytokinin biosynthesis and the activation of cytokinin degradation. These effects mediate the function of *AP1* in establishing determinate floral meristems in Arabidopsis (Han et al. 2014). In 35S:*AP1* Arabidopsis plants, extremely early flowering occurs after the production of five leaves, and the plants' primary shoot meristem has been converted into a compound terminal flower. The secondary shoot meristems present in the axils of cauline leaves have been transformed into solitary flowers. In addition, 35S:*AP1* Arabidopsis can partially complement the later flowering phenotype of a *lfy* mutant (Liljegren et al. 1999). Constitutive expression of the Arabidopsis *AP1* gene in juvenile citrus seedlings resulted in transgenic citrus plants with fertile flowers and fruits after just one year of growth. The transgenic citrus exhibited an appreciably shortened juvenile phase (Peña et al. 2001). In Populus, the overexpression of the Populus ortholog of *APETALA1 (LAP1)* produced a novel function in photoperiodic regulation of seasonal growth， the *LAP1* overexpression resulted in severe attenuation of SD-mediated growth cessation in hybrid aspen (Azeez et al. 2014).

However, the function analysis of *AP1* gene in Jatropha has not been reported. Currently, only one flowering-related gene, Jatropha *FLOWERING LOCUS T* (*JcFT*), has been functionally analyzed in Jatropha (Li et al. 2014). Overexpression of *JcFT* can produced more seeds in a shorter time frame by shortening the flowering time in Jatropha, suggesting the possibility to increase seed yield by manipulating the flowering time (Chen et al. 2014). Therefore, in this study, we cloned and characterized a Jatropha *AP1* homolog, *JcAP1*, through genetic complementation of the

Arabidopsis *AP1* loss-of-function mutant *ap1-11*. We analyzed the function of *JcAP1* in flowering induction and floral organ specification using transgenic Arabidopsis and Jatropha plants.

Materials and Methods

Plant materials and growth conditions

The roots, stems, mature leaves, inflorescence buds, flower buds, male flowers, female flowers and fruits of Jatropha were collected during the summer from Xishuangbanna, Yunnan Province, China. All of the tissues to be prepared for qRT-PCR were immediately frozen in liquid N_2 and stored at -80°C until use. The wild-type (WT) *Arabidopsis thaliana* Columbia ecotype (Col-0) and the *ap1-11* mutant of the same ecotype were purchased from The Arabidopsis Information Resource (TAIR) web site (http://www.arabidopsis.org/). The seeds of the Arabidopsis plants were germinated on 1/2 MS medium for one-week. Then, the seedlings were transferred to peat soil in plant growth chambers maintained at 22 ± 2 °C under long-day (16 light/8 h dark) or short-day (8 light/16 h dark) conditions. Phenotype analysis was performed on homozygous (T2) Arabidopsis plants and heterozygous (T0) Jatropha plants. More than 20 plants were used for the characterization of each Arabidopsis genotype. The number of rosette leaves and the number of days between transfer to soil and the first visible flower bud were recorded. The aboveground tissues of 15 d Arabidopsis seedlings were harvested to analyze mRNA transcription levels.

Cloning of JcAP1 cDNA

Total RNA was extracted from the Jatropha flower using the protocol described by Ding et al. (2008). First-strand cDNA was synthesized using M-MLV-reverse transcriptase according to the

manufacturer's instructions (TAKARA, Dalian, China). The full-length *JcAP1* genomic DNA sequence (Sato et al. 2011) (http://www.kazusa.or.jp/jatropha/) was amplified via PCR using the primers XK928 and XK929 (Table S1), which introduced *Kpn*I and *Sal*I recognition sites, respectively. The full-length *JcAP1* cDNA was amplified from flower cDNA with the same primers. The PCR products were subsequently cloned into the pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA) and sequenced. All primers used in this research were listed in Table S1

Sequence and phylogenetic analyses

JcAP1 amino acid sequence was deduced according to the coding sequence. Related sequences were identified through a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). To determine the amino acid identities, the alignment results were subjected to pairwise comparisons using DNAMAN 6.0. A phylogenetic tree based on the protein sequences was constructed with MEGA 5.0 (http://www.megasoftware.net). The amino acid sequences of AP1 were assembled using ClustalX. A neighbor-joining phylogenetic tree was generated with MEGA 5.0 using the Poisson model, with gamma-distributed rates and 1000 bootstrap replicates.

Plant expression vector construction and Arabidopsis and Jatropha transformation

To construct the 35S:*JcAP1* plant overexpression vector, the *JcAP1* sequence was excised from the pGEM-T vector (Promega, Corporation, Madison, Wisconsin, USA) using the restriction enzymes *Kpn*I and *Sal*I. Next, *JcAP1* was cloned into the pOCA30 vector containing the CaMV 35S promoter. Transformation of WT and *ap1-11* mutant plants with the *Agrobacterium* strain

144 EHA105 carrying the 35S:*JcAP1* construct was performed using the floral dip method (Clough &
145 Bent 1998). Transformation of Jatropha with the *Agrobacterium* strain EHA105 carrying the same
146 construct was performed according to the protocol described by Pan et al. (2010) and Fu et al.
147 (2015). All of the transgenic plants were confirmed using genomic PCR and RT-PCR.

148 Expression analysis via qRT-PCR

149 The roots, stems, mature leaves, inflorescence buds, flower buds, male flowers, female flowers
150 and fruits of mature Jatropha plants and the aboveground tissues of 15 days Arabidopsis seedlings
151 were collected for qRT-RCR detection. Total RNA was extracted from frozen Jatropha tissues as
152 described by Ding et al. (2008). Total RNA was extracted from frozen Arabidopsis tissues using
153 TRIzol reagent (Transgene, China). First-strand cDNA was synthesized with the PrimeScript® RT
154 Reagent Kit with gDNA Eraser (TAKARA, Dalian, China). The cDNA templates of first-strand
155 cDNA were diluted 5-fold with sterilized double-distilled water. qRT-PCR was performed using
156 SYBR® Premix Ex Taq™ II (TAKARA, Dalian, China) on a Roche 480 Real-Time PCR
157 Detection System (Roche, Mannheim, Germany). The primers employed for qRT-PCR are listed
158 in Table S1. qRT-PCR was conducted with three independent biological replicates and three
159 technical replicates for each sample. The data were analyzed using the $2^{-\Delta\Delta CT}$ method described
160 by Livak and Schmittgen (Livak & Schmittgen 2001). The transcript levels of specific genes were
161 normalized using Jatropha *ACTIN1* or Arabidopsis *ACTIN2*.

162 Results

163 Cloning and sequence analysis of *JcAP1*

A combined reverse transcriptase-polymerase chain reaction (RT-PCR) strategy was used to isolate *AP1*-like cDNA (*JcAP1*) from Jatropha. The *JcAP1* coding sequence (CDS) (GenBank accession no. KR013222) is comprised of 732 bp and encodes a 243-amino acid protein showing 81%, 79%, 75%, and 71% sequence identity to *Vitis vinifera* VvAP1 (Calonje et al. 2004), *Populus trichocarpa* PtAP1 (Tuskan et al. 2006), *Coffea arabica* CaAP1 (de Oliveira et al. 2014), and AtAP1 (Mandel et al. 1992), respectively.

The genomic sequence of *JcAP1* was 4928 bp and consisted of eight exons and seven introns, which resembles the genomic structure of the Arabidopsis *AP1* gene (Mandel et al. 1992). Multiple alignments were performed using the JcAP1 sequence and the sequences of AP1 homologs from other species. The MADS-box domain, K-Box domain, and euAP1 motif were determined (Fig. 1A).Since AP1 is closely related to CAL and FUL, and three genes exhibit high similarity and share redundant functions for floral meristem specification (Bowman et al. 1993), we undertook a phylogenetic analysis of the AP1/FUL MADS-box gene lineage (Fig. 1B), which is also called the *SQUA* lineage (Krogan & Ashton 2000). The phylogenetic tree is divided into two clades, AP1 clade and FUL clade. CAL, which appears only in *Brassicaceae*, is clustered in the AP1 clade (Litt & Irish 2003). Because CAL and AP1 originated from a recent duplication event <60 million years ago (Alvarez-Buylla et al. 2006). JcAP1 isolated in this study is clustered in the AP1 clade, while the JcFUL is clustered in the FUL clade. Moreover, JcAP1 is more closely related to AP1s of the *Euphorbiaceae* plants, such as *Ricinus communis* RcAP1, *Manihot esculenta* MeAP1 and *Plukenetia volubilis* PvAP1. In addition, the JcFUL is also closely related to *Ricinus communis* RcFUL (Fig. 1B). The results indicated that the AP1/FUL MADS-box gene phylogeny follows species phylogeny.

to interact with other MADS box proteins to confer sepal and petal identity (Mandel et al., 1992).

Expression pattern of JcAP1 in Jatropha

To assess the expression pattern of *JcAP1* in Jatropha, we performed a quantitative RT-PCR (qRT-PCR) analysis using RNA extracted from the roots, stems, mature leaves, inflorescence buds, flower buds, male flowers, female flowers and fruits. The morphologies of different developmental stages of flower were showed in Fig. S3. *JcAP1* was expressed in the inflorescence buds, flower buds, flowers, and fruits but showed very low expression in the roots, shoots and leaves. The expression profiles revealed that *JcAP1* was highly expressed during the later stages of inflorescence buds (IB3) and early stages of flower buds (FB1). During the development of inflorescences, the expression levels of *JcAP1* increased, whereas *JcAP1* expression decreased during the development of flower organs. In the reproductive organs, the fruits showed the lowest expression level of *JcAP1* (Fig. 2A). *JcAP1* was expressed in all floral organs, particularly in the sepals and petals (Fig. 2B).

Constitutive overexpression of *JcAP1* in Arabidopsis induces early flowering and abnormal flowers

To determine whether *JcAP1* is involved in the regulation of flowering time, *JcAP1* cDNA driven by the CaMV 35S promoter (Fig. 3A) was transformed into WT Arabidopsis. WT plants under the same growth conditions were used as a control. Transgenic plants were confirmed via qRT-PCR analysis of *JcAP1* expression using the aboveground tissues of 15 days Arabidopsis seedlings. Thirty-four independent T0 transgenic lines were generated with the 35S:*JcAP1* construct. Transgenic plants showed high *JcAP1* expression level (Fig. S1A). In the majority of the transgenic lines, bolting occurred notably earlier than in WT plants under both long-day (LD) and short-day (SD) conditions.

We examined the phenotypes of two independent homozygous transgenic lines (L12 and L30) in the T2 generation. Arabidopsis plants ectopically expressing *JcAP1*bolted 6–10 days earlier and produced 4–6 fewer rosette leaves than WT plants under LD conditions (Fig. 3B–D and Table 1). Under SD conditions, Arabidopsis plants ectopically expressing *JcAP1* flowered approximately 1–2 months earlier than WT (Fig. 3E–G and Table 2). Therefore, the overexpression of *JcAP1* in Arabidopsis significantly reduced the vegetative growth time.

In contrast to WT plants, the primary shoots of the transgenic plants were converted into compound terminal flowers consisting of two or three pistils surrounded by an abnormal number of sepals, petals, and stamens (Fig. 3I). Furthermore, the secondary shoots produced in cauline and rosette leaf axils were converted into solitary flowers. In extreme transgenic plants, all branches and inflorescences were replaced by solitary flowers (Fig. 3J).

Further analysis indicated that the promotion of flowering and abnormal terminal flowers in 35S:*JcAP1* transgenic Arabidopsis was correlated with a significant up-regulation of the floral meristem identity genes *AtLFY*, *AtFUL, AtAP1* and *AtCAL* and the floral organ identity genes *AtAGAMOUS* (*AtAG*)*, AtAP3* and *AtSEPs* (*AtSEP1, AtSEP2, AtSEP3*) (Fig. S1). The expression levels of these genes were highest in transgenic plants L12, whereas the *AtTFL1* expression level was slightly down-regulated (Fig. S1). Thus, L12 also showed the most obvious changes in phenotype of extremely early flowering and solitary and terminal flowers (Fig. 3). Thus, the phenotypes of the early-flowering and abnormal terminal flowers produced due to the ectopic expression of *JcAP1* in transgenic Arabidopsis were similar to those resulting from *AtAP1* overexpression (Mandel & Yanofsky 1995).

Constitutive overexpression of *JcAP1* in *ap1-11* mutant Arabidopsis induces early flowering and partially complements the phenotype

To further determine whether *JcAP1* can function similarly to *AtAP1*, the 35S:*JcAP1* construct (Fig. 3A) was transformed into Arabidopsis *ap1-11* mutant plants. Eight independent T0 transgenic lines were generated and confirmed through qRT-PCR analysis of *JcAP1* expression using RNA from aboveground tissues of 15 days Arabidopsis seedlings. WT and *ap1-11* mutants under the same growth conditions were used as controls. Most of the transgenic lines bolted earlier than the WT and *ap1-11* mutant plants under inductive LD conditions. The *ap1* mutants didn't exhibit significantly later flowering than WT (Table 3), which is because three homologous genes *AP1*, *CAL*, and *FUL* play redundant roles in control of flowering time. Each single mutant of *ap1, cal* or *ful* exhibited only slightly late flowering, whereas the triple mutant exhibited significantly late flowering (Ferrándiz et al. 2000).

To examine phenotypes, we selected two independent homozygous transgenic lines (C2 and C5) in the T2 generation that showed high *JcAP1*expression levels: line C2 and line C5 (Fig. 4I). Complementary transgenic lines C2 and C5 bolted 6–11 days earlier and produced 2–6 fewer rosette leaves than the controls under LD conditions (Fig. 4A–D and Table 3). In the extreme complementary transgenic line C5, solitary flowers appeared on the axils of rosette and cauline leaves and terminal flowers appeared on the primary shoots (Fig. 4D). The *ap1-11* mutant flowers lacked petals, and new secondary floral buds developed on the axils of the bract-like organs present

250 in the first whorl (Fig. 4F). The transgenic mutant C2 and C5 lines restored the development of
251 sepals and petals, and axillary flowers were rarely seen at the bracts (Fig. 4G and
252 H).Overexpression of *JcAP1* in *ap1* mutant leading to early flowering is because we used a strong
253 promoter, the *35S* promoter, which drives *JcAP1* constitutively expressing. Similar results were
254 found in Arabidopsis *ap1* mutant overexpressing the chrysanthemum and lily *AP1*-like genes
255 (Chen et al. 2008; Shchennikova et al. 2004). Further analysis indicated that the promotion of
256 flowering in the 35S:*JcAP1* transgenic Arabidopsis mutant was correlated with a significant up-
257 regulation of the flower meristem identity genes *AtLFY*, *AtSOC1* and *AtSEPs* (Fig. 4J and 4K).

258 These results demonstrate that the constitutive expression of *JcAP1* complements the defect
259 in floral organ development observed in the *ap1-11* mutant; thus, *JcAP1* functions as an A-class
260 gene in transgenic Arabidopsis.

261 Overexpression of *JcAP1* in Jatropha did not cause early flowering

262 Transgenic analysis performed in Arabidopsis suggested that *JcAP1* might act as a floral identity
263 gene in Jatropha. To test this hypothesis, we generated transgenic Jatropha with the 35S:*JcAP1*
264 construct (Fig. 3A) as previously described (Pan et al. 2010). Non-transgenic plants were used as
265 a control. Fifty-five independent transgenic lines were confirmed via PCR using genomic DNA
266 isolated from leaves of 2-month-old plantlets. And the partial results are shown in Fig. 5A. Next,
267 *JcAP1* expression levels in fourteen PCR-positive lines were examined through qRT-PCR using
268 RNA extracted from young leaves of 2-month-old plantlets (Fig. 5B). To our surprise, all of these

transgenic Jatropha lacked an early-flowering phenotype (Fig. 6A and B). When regenerated plantlets were grown in the field for 4 months, flower buds emerged in both transgenic and control plants (Fig. 6C–H). We chose L2 and L20, which exhibited high and intermediate expression levels, respectively (Fig. 5B), to further analyze the expression levels of several floral identity-related genes in the shoot apices of 6-month-old plantlets. The results showed that the transcript levels of *JcLFY*, *JcSOC1* and *JcTFL1s* (Fig. S2) were not significantly altered in both transgenic lines. The 35S promoter was highly active in the Jatropha inflorescence buds (Tao et al. 2015), but the inflorescence structure (Fig. 6 D, F, H) and floral organ pattern (Fig. 6I) were not obviously different. These results indicate that *JcAP1* may be inadequate to promote flowering and floral organ development by itself in Jatropha.

Discussion

Recently, Jatropha has garnered significant attention as a potential oilseed plant for the production of renewable biofuel. Despite the interest in this woody oil plant, relatively little is known regarding the molecular biology of this species compared with more established oilseed crops, such as rapeseed (Handa 2003; Kresovich et al. 1995) and castor bean (Chan et al. 2010).

Amino acid sequence similarity, protein structures, and phylogenetic analysis suggested that JcAP1 exhibits a similar function to other AP1 homologues. In Arabidopsis, *AP1* functions redundantly with *FUL* in specifying floral meristem identity (Bowman et al. 1993). However, in the phylogenetic tree, AP1 and FUL clustered in the separate clades indicated the functional divergence between the two paralogs. Except for floral meristem determination, *FUL* is required for proper fruit and leaf development in Arabidopsis (Gu et al. 1998), and *FUL* prevents normal

290 senescence and winter dormancy in woody *Populus tremula* L (Hoenicka et al. 2008). JcAP1 was
291 clustered in the AP1 clade suggesting this gene may functions similarly as other AP1 genes in the
292 regulation of flowering and flower organ development.

293 Quantitative RT-PCR results showed that *JcAP1* transcripts were highly expressed in
294 inflorescence buds, flower buds, sepals and petals (Fig. 2). This expression pattern is consistent
295 with that of *AtAP1* in Arabidopsis (Mandel et al. 1992). The expression profiles revealed that the
296 highest *JcAP1* transcript levels occurred in the earlier stages of male and female flower buds (Fig.
297 2A), implying that *JcAP1* may play a role in maintaining the normal development of flower
298 patterns (Collaudin 2012). In addition, the high *JcAP1* expression levels observed in sepals and
299 petals (Fig. 2B) suggested that *JcAP1* may be involved in sepal and petal development. In
300 Arabidopsis, the function of *AP1* in the regulation of sepal and petal development was revealed
301 using an *ap1* mutant (Mandel et al. 1992); The *AP1* gene was identified as a member of the floral
302 meristem identity genes, which largely acts downstream of the floral integrators *FT*, *LFY*, and
303 *SOC1* (Bowman et al. 1993; Liljegren et al. 1999; Liu et al. 2013; Riechmann et al. 1996). In this
304 study, we showed that overexpression of *JcAP1* in Arabidopsis resulted in reduced vegetative
305 growth, early flowering and the formation of terminal and solitary flowers (Fig. 3; Tables 1, 2).
306 These findings are similar to the phenotypic changes caused by constitutive expression of *AP1*
307 homologs in Arabidopsis (Chi et al. 2011; Kotoda et al. 2002; Liljegren et al. 1999; Liu et al. 2013;
308 Mandel & Yanofsky 1995; Sun et al. 2014; Wang et al. 2013; Weigel & Nilsson 1995;
309 Winterhagen et al. 2013). The production of terminal and solitary flowers in *AP1*-overexpressing
310 plants is due to the inhibition of *TFL1* expression induced by *AP1* (Blazquez et al. 2006). In
311 Arabidopsis, young seedlings showed weak *TFL1* expression; the *TFL1* expression increased after
312 8 days and young inflorescences showed the strongest *TFL1* expression (Bradley et al. 1997). In

313 previous research, *TFL1* down-regulated directly by *AP1* was confirmed by the CHIP-Seq analysis
314 (Kaufmann et al. 2010). Compared with WT plants, the *TFL1* expression was not detected in
315 primary shoot apices and secondary meristems in *AP1* overexpressing plants.(Liljegren et al.
316 1999). Similarly, in our research, when *JcAP1*was overexpressed in Arabidopsis, the expression
317 of *TFL1* was also suppressed. The aboveground tissues of 15 days plants were used for detecting
318 the *TFL1* expression in our study. The inflorescence buds had appeared in *JcAP1*overexpressing
319 plants while WT plants were still in vegetative growth. According to the *TFL1* expression pattern
320 in Arabidopsis, in which the inflorescences showed the highest expression level, *TFL1* expression
321 in transgenic plant was supposed to be higher than in WT plants. However, the expression level of
322 *TFL1* was decreased in the *JcAP1* highly expressing line (L12) (Fig. S1L). This result indicated
323 that the increase of *JcAP1*expression repressed the *TFL1* expression. Consistently, when *JcAP1*
324 was lowly expressed (L30), the expression level of *TFL1* was markedly increased (Fig. S1L). In
325 Jatropha, nevertheless, the expression of three *JcTFL1s* (*JcTFL1a*, *JcTFL1b* and *JcTFL1c*) were
326 not significantly altered in *JcAP1*overexpressing plants (Fig. S2D). Thus, we supposed that
327 *JcTFL1* was indirectly relative to *JcAP1*.

328 Overexpression of *JcAP1* in the Arabidopsis *ap1-11* mutant resulted in early flowering,
329 restored sepal and petal development, and repression of secondary flower formation in the bract
330 axils (Fig. 5I–K). These results are consistent with the phenotypic changes observed following the
331 ectopic expression of Chrysanthemum and lily *AP1*-like genes in the Arabidopsis *ap1* mutant
332 (Chen et al. 2008; Shchennikova et al. 2004). These findings imply that *JcAP1* acts as a functional
333 homolog of *AP1* in Arabidopsis.

334 In contrast to *JcAP1*-overexpressing Arabidopsis, *JcAP1*-overexpressing Jatropha did not
335 exhibit early flowering (Fig. 6). But the expression profile showed that *JcAP1* was predominantly

expressed in inflorescence buds and flower buds (Fig. 2A). It indicates that *JcAP1* may be insufficient to regulate flowering time or floral organ development by itself in Jatropha. Similar to our findings, overexpression of the Populus ortholog of *APETALA1*in Arabidopsis led to early flowering whereas it failed to promote flowering in hybrid aspen (Azeez et al. 2014). In addition new functions of *AP1* homologs have been identified in several species. The *AP1* homolog of grapevine (*VAP1*) is involved in the formation of tendrils (Calonje et al. 2004). Wheat *AP1* (*WAP1*) has no known role in flower development but is required for vernalization and phase transition (Danyluk et al. 2003; Handa 2003; Trevaskis et al. 2003). In tomato plants, *AP1/FUL* MADS box genes are involved in tomato leaf development (Burko et al. 2013).

The molecular mechanisms controlling flowering in perennials have not been studied as extensively as those of annual plants (Albani & Coupland 2010). It seems more complex in perennial plants in that a well-known identified flowering gene is unable to control the flowering time. For example, overexpression of *LFY* failed to cause early flowering in apple (Flachowsky et al. 2010) and hybrid populous (Rottmann et al. 2000). Overexpression of miR172, which caused extremely early flowering in Arabidopsis (Aukerman & Sakai 2003), was unable to promote flowering in the perennial plant *Cardamine flexuosa* unless it was treated with vernalization simultaneously (Zhou et al. 2013). In this study, the results also suggest that *JcAP1* itself may not be sufficient to promote flowering in Jatropha; perhaps it needs to be associated with other factors to function in these processes. Other important floral identity genes or environment factors need to be characterized in Jatropha in the future study.

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Abbreviations

The following abbreviations are used in this manuscript:

AG: AGAMOUS

AP1: APETALA 1

AP3: APETALA 3

CAL: CAULIFLOWER

CaMV: Cauliflower Mosaic Virus

FT: FLOWERING LOCUS T

FUL: FRUITFULL

LD: Long day condition

qRT-PCR: Quantitative reverse transcriptase-polymerase chain reaction

SD: Short day condition

SEP: SEPALLATA

SOC1: *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*

TFL1: TERMINAL FLOWER 1

References

Akashi K. 2012. *Jatropha* research: A new frontier for biofuel development. *Plant Biotechnology* 29:121.

Albani MC, and Coupland G. 2010. Comparative analysis of flowering in annual and perennial plants. In: Marja CPT, ed. *Current Topics in Developmental Biology*: Academic Press, 323-348.

Alvarez-Buylla ER, Garcia-Ponce B, and Garay-Arroyo A. 2006. Unique and redundant functional domains of *APETALA1* and *CAULIFLOWER*, two recently duplicated *Arabidopsis thaliana* floral MADS-box genes. *Journal of Experimental Botany* 57:3099-3107. 10.1093/jxb/erl081

Aukerman MJ, and Sakai H. 2003. Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *Plant Cell* 15:2730-2741.

Azeez A, Miskolczi P, Tylewicz S, and Bhalerao R. 2014. A tree ortholog of *APETALA1* mediates photoperiodic control of seasonal growth. *Current Biology* 24:717.

Blazquez MA, Ferrandiz C, Madueno F, and Parcy F. 2006. How floral meristems are built. *Plant Molecular Biology* 60:855-870.

Bowman JL, Alvarez J, Weigel D, Meyerowitz EM, and Smyth DR. 1993. Control of flower development in *Arabidopsis thaliana* by *APETALA 1* and interacting genes. *Development* 119:721-721.

Bradley D, Ratcliffe O, Vincent C, Carpenter R, and Coen E. 1997. Inflorescence commitment and architecture in *Arabidopsis*. *Science* 275:80-83.

Burko Y, Shleizer-Burko S, Yanai O, Shwartz I, Zelnik ID, Jacob-Hirsch J, Kela I, Eshed-Williams L, and Ori N. 2013. A role for APETALA1/FRUITFULL transcription factors in tomato leaf development. *Plant Cell* 25:2070-2083.

Calonje M, Cubas P, Martínez-Zapater JM, and Carmona MJ. 2004. Floral meristem identity genes are expressed during tendril development in *grapevine*. *Plant Physiol* 135:1491-1501.

Chan AP, Crabtree J, Zhao Q, Lorenzi H, Orvis J, Puiu D, Melake-Berhan A, Jones KM, Redman J, and Chen G. 2010. Draft genome sequence of the oilseed species *Ricinus communis*. *Nature Biotechnology* 28:951-956.

Chen A, Li C, Hu W, Lau MY, Lin H, Rockwell NC, Martin SS, Jernstedt JA, Lagarias JC, and Dubcovsky J. 2014. PHYTOCHROME C plays a major role in the acceleration of wheat flowering under long-day photoperiod. *Proceedings of the National Academy of Sciences* 111:10037-10044. 10.1073/pnas.1409795111

Chen M-K, Lin I-C, and Yang C-H. 2008. Functional analysis of three lily (*Lilium longiflorum*) APETALA1-like MADS box genes in regulating floral transition and formation. *Plant cell Physiology* 49:704-717.

Chi Y, Huang F, Liu H, Yang S, and Yu D. 2011. An *APETALA1*-like gene of soybean regulates flowering time and specifies floral organs. *Journal of Plant Physiology* 168:2251-2259.

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

Collaudin S. 2012. Morphogenesis of the flower of Arabidopsis, genes networks and mathematical modelling. *Organ* 2012:10-01.

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, and Sarhan F. 2003. TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. *Plant Physiology* 132:1849-1860.

de Oliveira RR, Cesarino I, Mazzafera P, and Dornelas MC. 2014. Flower development in Coffea arabica L.: new insights into MADS-box genes. *Plant Reproduction* 27:79-94.

Ding L-W, Sun Q-Y, Wang Z-Y, Sun Y-B, and Xu Z-F. 2008. Using silica particles to isolate total RNA from plant tissues recalcitrant to extraction in guanidine thiocyanate. *Analytical Biochemistry* 374:426-428. 10.1016/j.ab.2007.11.030

Divakara B, Upadhyaya H, Wani S, and Gowda C. 2010. Biology and genetic improvement of Jatropha curcas L.: A review. *Applied Energy* 87:732-742.

Ferrándiz C, Gu Q, Martienssen R, and Yanofsky MF. 2000. Redundant regulation of meristem identity and plant architecture by *FRUITFULL, APETALA1* and *CAULIFLOWER*. *Development* 127:725-734.

Flachowsky H, Haettasch C, Hoefer M, Peil A, and Hanke M-V. 2010. Overexpression of *LEAFY* in apple leads to a columnar phenotype with shorter internodes. *Planta* 231:251-263. 10.1007/s00425-009-1041-0

Fu Q, Li C, Tang M, Tao Y-B, Pan B-Z, Zhang L, Niu L, He H, Wang X, and Xu Z-F. 2015. An efficient protocol for *Agrobacterium*-mediated transformation of the biofuel plant *Jatropha curcas* by optimizing kanamycin concentration and duration of delayed selection. *Plant Biotechnology Reports* 9:405-416. 10.1007/s11816-015-0377-0

Ghosh A, Chaudhary DR, Reddy MP, Rao SN, Chikara J, Pandya JB, Patolia JS, Gandhi MR, Adimurthy S, Vaghela N, Mishra S, Rathod MR, Prakash AR, Shethia BD, Upadhyay SC, Balakrishna V, Prakash R, and Ghosh PK. 2007. Prospects for jatropha methyl ester (biodiesel) in India. *International Journal of Environmental Studies* 64:659-674. 10.1080/00207230701766499

Ghosh A, Chikara J, Chaudhary DR, Prakash A, Boricha G, and Zala A. 2010. Paclobutrazol arrests vegetative growth and unveils unexpressed yield potential of *Jatropha curcas*. *Journal of Plant Growth Regulation* 29:307-315. 10.1007/s00344-010-9137-0

Gu K, Tian D, Mao H, Wu L, and Yin Z. 2015. Development of marker-free transgenic *Jatropha curcas* producing curcin-deficient seeds through endosperm-specific RNAi-mediated gene silencing. *BMC Plant Biol* 15:242-251.

Gu Q, Ferrandiz C, Yanofsky MF, and Martienssen R. 1998. The *FRUITFULL MADS*-box gene mediates cell differentiation during *Arabidopsis* fruit development. *Development* 125:1509-1517.

Han Y, Zhang C, Yang H, and Jiao Y. 2014. Cytokinin pathway mediates *APETALA1* function in the establishment of determinate floral meristems in *Arabidopsis*. *Proceedings of the National Academy of Sciences of The United States of America* 111:6840-6845.

Handa H. 2003. The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. *Nucleic acids research* 31:5907-5916.

Hirakawa H, Tsuchimoto S, Sakai H, Nakayama S, Fujishiro T, Kishida Y, Kohara M, Watanabe A, Yamada M, and Aizu T. 2012. Upgraded genomic information of *Jatropha curcas* L. *Plant Biotechnology* 29:123-130.

Hoenicka H, Nowitzki O, Hanelt D, and Fladung M. 2008. Heterologous overexpression of the birch *FRUITFULL*-like MADS-box gene *BpMADS4* prevents normal senescence and winter dormancy in Populus tremula L. *Planta* 227:1001-1011. 10.1007/s00425-007-0674-0

Irish VF, and Sussex IM. 1990. Function of the apetala-1 gene during *Arabidopsis* floral development. *Plant Cell* 2:741-753.

Kajikawa M, Morikawa K, Inoue M, Widyastuti U, Suharsono S, Yokota A, and Akashi K. 2012. Establishment of bispyribac selection protocols for *Agrobacterium tumefaciens*-and *Agrobacterium rhizogenes*-mediated transformation of the oil seed plant *Jatropha curcas* L. *Plant Biotechnology* 29:145-153.

Kaufmann K, Wellmer F, Muino JM, Ferrier T, Wuest SE, Kumar V, Serrano-Mislata A, Madueno F, Krajewski P, Meyerowitz EM, Angenent GC, and Riechmann JL. 2010. Orchestration of Floral Initiation by *APETALA1*. *Science* 328:85-89. 10.1126/science.1185244

Khalil HPSA, Aprilia NAS, Bhat AH, Jawaid M, Paridah MT, and Rudi D. 2013. A *Jatropha* biomass as renewable materials for biocomposites and its applications. *Renew Sust Energ Rev* 22:667-685. http://dx.doi.org/10.1016/j.rser.2012.12.036

King AJ, Montes LR, Clarke JG, Itzep J, Perez CA, Jongschaap RE, Visser RG, van Loo EN, and Graham IA. 2015. Identification of QTL markers contributing to plant growth, oil yield and fatty acid composition in the oilseed crop *Jatropha curcas* L. *Biotechnol Biofuels* 8:1-17.

Kotoda N, Wada M, Kusaba S, Kano-Murakami Y, Masuda T, and Soejima J. 2002. Overexpression of *MdMADS5*, an *APETALA1*-like gene of apple, causes early flowering in transgenic *Arabidopsis*. *Plant Science* 162:679-687.

Kresovich S, Szewc-McFadden A, Bliek S, and McFerson J. 1995. Abundance and characterization of simple-sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* L.(rapeseed). *Theoretical and Applied Genetics* 91:206-211.

492 Krogan NT, and Ashton NW. 2000. Ancestry of plant MADS-box genes revealed by bryophyte
493 (Physcomitrella patens) homologues. *New Phytologist* 147:505-517. 10.1046/j.1469-
494 8137.2000.00728.x

495 Kumar N, Anand KGV, Pamidimarri DVNS, Sarkar T, Reddy MP, Radhakrishnan T, Kaul T,
496 Reddy MK, and Sopori SK. 2010. Stable genetic transformation of Jatropha curcas via
497 Agrobacterium tumefaciens-mediated gene transfer using leaf explants. *Industrial Crops*
498 *and Products* 32:41-47. DOI 10.1016/j.indcrop.2010.03.002

499 Li C, Luo L, Fu Q, Niu L, and Xu Z-F. 2014. Isolation and functional characterization of *JcFT*, a
500 *FLOWERING LOCUS T (FT)* homologous gene from the biofuel plant *Jatropha curcas*.
501 *BMC Plant Biology* 14. 10.1186/1471-2229-14-125

502 Liljegren SJ, Gustafson-Brown C, Pinyopich A, Ditta GS, and Yanofsky MF. 1999. Interactions
503 among *APETALA1, LEAFY*, and *TERMINAL FLOWER1* specify meristem fate. *Plant Cell*
504 11:1007-1018.

505 Litt A, and Irish VF. 2003. Duplication and diversification in the *APETALA1/FRUITFULL* floral
506 homeotic gene lineage: implications for the evolution of floral development. *Genetics*
507 165:821-833.

508 Liu L, Zhu Y, Shen L, and Yu H. 2013. Emerging insights into florigen transport. *Curr Opin Plant*
509 *Biol* 16:607-613. 10.1016/j.pbi.2013.06.001

510 Livak KJ, and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time
511 quantitative PCR and the 2 $^{\Delta\Delta C\ T}$ method. *Methods* 25:402-408.

512 Mandel MA, Gustafson-Brown C, Savidge B, and Yanofskay MF. 1992. Molecular
513 chanracterization of the *Arbidopsis* floral homeotic gene *APETALA1*. *Nature* 360:5.

514 Mandel MA, and Yanofsky MF. 1995. A gene triggering flower formation in *Arabidopsis*. *Nature*
515 377:522-524.

516 Mao HZ, Ye J, and Chua NH. 2013. Genetic transformation of *Jatropha curcas*. Joil (S) Pte Ltd.

517 Misra P, Toppo DD, Mishra MK, Saema S, and Singh G. 2012. *Agrobacterium tumefaciens*-
518 mediated transformation protocol of *Jatropha curcas* L. using leaf and hypocotyl segments.
519 *Journal of Plant Biochemistry and Biotechnology* 21:128-133.

520 Mofijur M, Rasul M, Hyde J, Azad A, Mamat R, and Bhuiya M. 2016. Role of biofuel and their
521 binary (diesel–biodiesel) and ternary (ethanol–biodiesel–diesel) blends on internal
522 combustion engines emission reduction. *Renew Sust Energ Rev* 53:265-278.

523 Ng M, and Yanofsky MF. 2001. Activation of the *Arabidopsis* B class homeotic genes by
524 *APETALA1*. *Plant Cell* 13:739-753.

525 Ong H, Mahlia T, Masjuki H, and Norhasyima R. 2011. Comparison of *palm* oil, *Jatropha curcas*
526 and *Calophyllum inophyllum* for biodiesel: a review. *Renewable & Sustainable Energy*
527 *Reviews* 15:3501-3515.

528 Pan B-Z, and Xu Z-F. 2011. Benzyladenine treatment significantly increases the seed yield of the
529 biofuel plant *Jatropha curcas*. *J Plant Growth Regul* 30:166-174.

Pan J, Fu Q, and Xu Z-F. 2010. *Agrobacterium tumefaciens*-mediated transformation of biofuel plant *Jatropha curcas* using kanamycin selection. *African journal of agricultural research* 9:6477-6481.

Pandey VC, Singh K, Singh JS, Kumar A, Singh B, and Singh RP. 2012. *Jatropha curcas*: A potential biofuel plant for sustainable environmental development. *Renewable & Sustainable Energy Reviews* 16:2870-2883.

Peña L, Martín-Trillo M, Juárez J, Pina JA, Navarro L, and Martínez-Zapater JM. 2001. Constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes in citrus reduces their generation time. *Nature Biotechnology* 19:263-267.

Pramanik K. 2003. Properties and use of jatropha curcas oil and diesel fuel blends in compression ignition engine. *Renewable Energy* 28:239-248. http://dx.doi.org/10.1016/S0960-1481(02)00027-7

Pua F-l, Fang Z, Zakaria S, Guo F, and Chia C-h. 2011. Direct production of biodiesel from high-acid value *Jatropha* oil with solid acid catalyst derived from lignin. *Biotechnology for Biofuels* 4:1-8.

Riechmann JL, Krizek BA, and Meyerowitz EM. 1996. Dimerization specificity of *Arabidopsis MADS* domain homeotic proteins *APETALA1, APETALA3, PISTILLATA,* and *AGAMOUS*. *Proceedings of the National Academy of Sciences of The United States of America* 93:4793-4798.

Rottmann WH, Meilan R, Sheppard LA, Brunner AM, Skinner JS, Ma CP, Cheng SP, Jouanin L, Pilate G, and Strauss SH. 2000. Diverse effects of overexpression of *LEAFY* and *PTLF*, a poplar (Populus) homolog of *LEAFY/FLORICAULA*, in transgenic poplar and Arabidopsis. *Plant J* 22:235-245. 10.1046/j.1365-313x.2000.00734.x

Sato S, Hirakawa H, Isobe S, Fukai E, Watanabe A, Kato M, Kawashima K, Minami C, Muraki A, Nakazaki N, Takahashi C, Nakayama S, Kishida Y, Kohara M, Yamada M, Tsuruoka H, Sasamoto S, Tabata S, Aizu T, Toyoda A, Shin-i T, Minakuchi Y, Kohara Y, Fujiyama A, Tsuchimoto S, Kajiyama Si, Makigano E, Ohmido N, Shibagaki N, Cartagena JA, Wada N, Kohinata T, Atefeh A, Yuasa S, Matsunaga S, and Fukui K. 2011. Sequence analysis of the genome of an oil-bearing tree, *Jatropha curcas* L. *DNA Res* 18:65-76.

Shchennikova AV, Shulga OA, Immink R, Skryabin KG, and Angenent GC. 2004. Identification and characterization of four chrysanthemum MADS-box genes, belonging to the APETALA1/FRUITFULL and SEPALLATA3 subfamilies. *Plant Physiology* 134:1632-1641.

Sinha P, Islam MA, Negi MS, and Tripathi SB. 2015. Changes in oil content and fatty acid composition in *Jatropha curcas* during seed development. *Ind Crop Prod* 77:508-510.

Sun L-M, Zhang J-Z, Mei L, and Hu C-G. 2014. Molecular cloning, promoter analysis and functional characterization of *APETALA 1*-like gene from precocious trifoliate orange (*Poncirus trifoliata*). *Scientia Horticulturae* 178:95-105.

Tao Y-B, He L-L, Niu L-J, and Xu Z-F. 2015. Isolation and characterization of an ubiquitin extension protein gene (JcUEP) promoter from Jatropha curcas. *Planta* 241:823-836. 10.1007/s00425-014-2222-z

Tjeuw J, Slingerland M, and Giller K. 2015. Relationships among *Jatropha curca*s seed yield and vegetative plant components under different management and cropping systems in Indonesia. *Biomass Bioenerg* 80:128-139.

Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, and Dennis ES. 2003. MADS box genes control vernalization-induced flowering in cereals. *Proceedings of the National Academy of Sciences of the United States of America* 100:13099-13104.

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, and Salamov A. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313:1596-1604.

Wagner D, Sablowski RWM, and Meyerowitz EM. 1999. Transcriptional activation of *APETALA1* by *LEAFY*. *Science* 285:582.

Wang J, Zhang X, Yan G, Zhou Y, and Zhang K. 2013. Over-expression of the *PaAP1* gene from sweet cherry (*Prunus avium L*.) causes early flowering in *Arabidopsis thaliana*. *Journal of Plant Physiology* 170:315-320.

Weigel D, and Nilsson O. 1995. A developmental switch sufficient for flower initiation in diverse plants. *Nature* 377:495-500.

William DA, Su YH, Smith MR, Lu M, Baldwin DA, and Wagner D. 2004. Genomic identification of direct target genes of LEAFY. *P Natl Acad Sci USA* 101:1775-1780. 10.1073/pnas.0307842100

Winterhagen P, Tiyayon P, Samach A, Hegele M, and Wünsche J. 2013. Isolation and characterization of *FLOWERING LOCUS T* subforms and *APETALA1* of the subtropical fruit tree *Dimocarpus longan*. *Plant Physiology and Biochemistry* 71:184.

Wu J, Liu Y, Tang L, Zhang F, and Chen F. 2011. A study on structural features in early flower development of *Jatropha curcas L.* and the classification of its inflorescences. *African journal of agricultural research* 6:275-284.

Wu PZ, Zhou CP, Cheng SF, Wu ZY, Lu WJ, Han JL, Chen YB, Chen Y, Ni PX, Wang Y, Xu X, Huang Y, Song C, Wang ZW, Shi N, Zhang XD, Fang XH, Yang Q, Jiang HW, Chen YP, Li MR, Wang Y, Chen F, Wang J, and Wu GJ. 2015. Integrated genome sequence and linkage map of physic nut (*Jatropha curcas* L.), a biodiesel plant. *Plant Journal* 81:810-821. Doi 10.1111/Tpj.12761

Zhou C-M, Zhang T-Q, Wang X, Yu S, Lan H, Tang H, Feng Z-Y, Zozomova-Lihova J, and Wang J-W. 2013. Molecular Basis of Age-Dependent Vernalization in Cardamine flexuosa. *Science* 340:1097-1100. 10.1126/science.1234340

606 **Tables**

607 **Table 1** Overexpression of *JcAP1* promotes flowering in Arabidopsis under LD conditions.

Lines	N	Rosette leaves	Flower bud formation time(day)
WT	22	10.14 ± 0.89	20.18 ± 0.73
Line 12	20	3.95 ± 0.51**	10.60 ± 1.23**
Line 30	20	5.40 ± 0.99**	14.25 ± 1.37**

608 WT plants and two independent *JcAP1*-overexpressing lines (L12 and L30) grown under LD
609 conditions (16 h light/8 h dark) were subjected to the analysis of rosette leaves and flowering
610 times. N = plant number. The rosette leaves and flowering times are presented as the mean ±
611 standard deviation. **significantly different from the control at the 1% level.

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Table 2 Overexpression of *JcAP1* promotes flowering in Arabidopsis under SD conditions.

Lines	N	Rosette leaves	Flower bud formation time(day)
WT	22	60.41 ± 3.95	104.00 ± 5.83
Line 12	25	14.68 ± 1.44**	33.72 ± 3.06**
Line 30	27	31.63 ± 2.50**	58.33 ± 3.95**

WT plants and two independent *JcAP1*-overexpressing lines (L12 and L30) grown under SD growing conditions (8 h light/16 h dark) were subjected to the analysis of rosette leaves and flowering times. N = plant number. The rosette leaves and flowering times are presented as the mean ± standard deviation. **significantly different from the control at the 1% level.

637 **Table 3** Overexpression of *JcAP1* in *ap1-11* Arabidopsis plants promotes flowering time under
638 LD conditions.

Lines	N	Rosette leaves	Flower bud formation time(day)
WT	25	10.54 ± 0.92	20.18 ± 0.73
ap1-11	20	11.02 ± 0.87	20.80 ± 1.23
Line C2	15	8.40 ± 0.99**	14.05 ± 1.45**
Line C5	15	3.95 ± 0.51**	9.80 ± 1.18**

639 WT plants, the *ap1-11* mutant, and two independent *JcAP1*-overexpressing lines (C2 and C5)
640 grown under LD growing conditions (16 h light/8 h dark) were subjected to the analysis of
641 rosette leaves and flowering times. N = plant number. The rosette leaves and flowering times
642 are presented as the mean ± standard deviation. **significantly different from the control at the
643 1% level.

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657 **Supporting Information**

658 **Table S1 Primers used for all experiments**

Target template	Primer name	Primer sequence
JcAP1 full length	XK928-KpnI F	GGGGTACCCCGGAAAGAAGAGGAAAAATTTATACA
	XK929-SalI R	GCGTCGACGTAGCATGTAGTAATCTCTCTCCTGTT
JcAP1 qRT-PCR	XA311 F	TAACAGACTCAAGGCGAAGGT
	XA312 R	AGTTGGTTGTTTCTTGCTCGG
JcLFY qRT-PCR	XT655 F	GGATAAGATACTACACAGCAGCGA
	XT656 R	TAACCCTTCTTGAGAGAGAGCATC
JcSOC1 qRT-PCR	XK656 F	TTCTTGGACGGCAACGCTTA
	XK657 R	CTCTCGGAAAAGTGTGGGATC
JcTFL1a qRT-PCR	XA520 F	GTGTATGTTAGTACCGTATTTGGAT
	XA521 R	CTAAACCAAAGAGCTTATTCTAGGC
JcTFL1b qRT-PCR	XA485 F	ACCAGTAGACCCTCTTATTGTTGAGA
	XA486 R	TCATATCATCTCCTTCCACAGCAACT
JcTFL1c qRT-PCR	XA203 F	ACGGAGCCACAGCCACTTACTGTAG
	XA204 R	ACTCTAGGTTTAGCAGCAATGACCG
AtAP1 qRT-PCR	XT803 F	TTTGGAGAGAAACCAGAGGCATT
	XT804 R	GTAAGGATGCTGGATTTGGTGCT
AtLFY qRT-PCR	XT805 F	TGCTCTCTCCCAAGAAGGGTTAT
	XT806 R	TTGGTTTCTTTCTCCGTCTCTGC
AtAP3 qRT-PCR	XT896 F	GTCTTGAGGATGAAATGGAAAAC
	XT897 R	TGGTATCCAAGAACTGAGTCGTA
AtAG qRT-PCR	XT898 F	GCGTCAACAAATAATCAGCATAC
	XT899 R	CGAAGAATCTGGTTATCGTTATG
AtFLC qRT-PCR	XT900 F	CTTTCTGTTCTCTGTGACGCATC
	XT901 R	AGTCTCAAGGTGTTCCTCCAGTT
AtFUL qRT-PCR	XT902 F	GCTATCAAGAGCATTAGGTCAAG
	XT903 R	GTTACGCAGTATTGAGGCAGAA
AtCAL qRT-PCR	XT904 F	GGAGAGAAACCAAAGGCATTATC
	XT905 R	TCCTTTCTTTGGAGGTGGTTGA
AtTFL1 qRT-PCR	XT906 F	ATAATGGGGAGAGTGGTAGGAGA
	XT907 R	TCTGGGTCTATCATCACCAAAGT
AtSEP1 qRT-PCR	XK904 F	CTTCTTGGGGAGGATTTAGGA
	XK905 R	ACATTCTGTTCACCACCTTCC
AtSEP2 qRT-PCR	XK906 F	GGACATCCTCAGGCTCATTCTC
	XK907 R	AGAAGTATCGCTCACAGCATCC
AtSEP3 qRT-PCR	XK908 F	AATGGGAAGAGGGAGAGTAGA
	XK909 R	TTCTGGTGCTCCATAGTTACA
AtFT qRT-PCR	XA358 F	AGAGGTGACTAATGGCTTGGAT
	XA359 R	AAGGTTGTTCCAGTTGTAGCAG

JcACTIN1 qRT-PCR	XK191 F	CTCCTCTCAACCCCAAAGCCAA
	XK192 R	CACCAGAATCCAGCACGATACCA
AtACTIN2 qRT-PCR	XK718 F	TGTGCCAATCTACGAGGGTTT
	XK719 R	TTTCCCGCTCTGCTGTTGT

Figure S1 Quantitative RT-PCR analysis of *JcAP1* and other flower-related genes in WT and transgenic Arabidopsis. (A) The expression level of *JcAP1* in WT and transgenic Arabidopsis L12 L30 plants; *JcAP1* expression was not detected in WT; transcript levels were not normalized. (B-L) The expression levels of *AtLFY*, *AtAP1*, *AtFUL*, *AtAG*, *AtAP3*, *AtSEP1*, *AtSEP2*, *AtSEP3*, *AtFT*, *AtCAL,* and *AtTFL1*, respectively. RNA sample extracted from apex and rosette leaves of 35S:*JcAP1* transgenic and WT plants cultured for 15 days in a pot. Transcript levels were normalized using the *AtACTIN2* gene as a reference. The mRNA level in WT was set as the standard, with a value of 1.

Figure S2 Quantitative RT-PCR analysis of *JcAP1* and flower-related genes in WT and 35S:*JcAP1* transgenic Jatropha. The expression levels of *JcAP1*, *JcLFY*, *JcSOC1*, and *JcTFL1s* were detected in shoot apices of 6-month-old plantlets of WT and transgenic Jatropha. The qRT-PCR results were obtained using two independent biological replicates and three technical replicates for each RNA sample extracted from the apex of the 35S:*JcAP1* transgenic and WT shoots. Transcript levels were normalized using the *JcACTIN1* gene as a reference. The mRNA level in WT was set as the standard, with a value of 1.

Figure S3 Flower morphological characteristics of Jatropha in different developmental stages. (A) Inflorescence bud stage 1 (IB1): 0-5 days, inflorescence buds are visible; (B) inflorescence bud stage 2 (IB2): 1 week after IB1; (C) inflorescence bud stage 3 (IB3): 1 week after IB2; (D) flower bud stage 1 (FB1): 1 week after IB3; (E) flower bud stage 2 (FB2): male flower buds (MFB) and female flower buds (FFB) are identifiable one week after FB1; (F): male and female flower stage: male flowers (MF) and female flowers

682 (FF) bloomed one week after FB2. In (E) and (F), red arrows indicate FFBs and FFs,

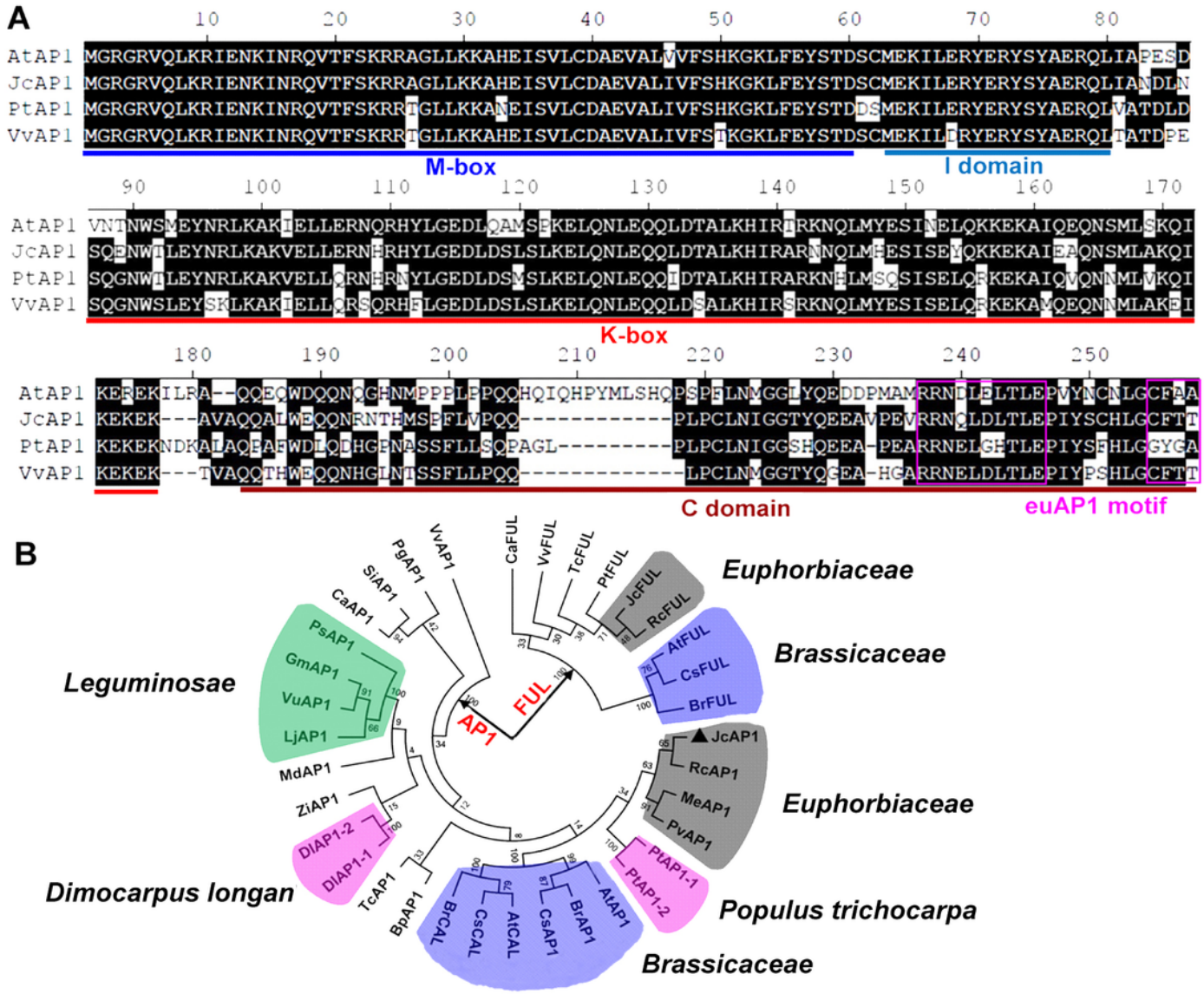
683 respectively; and pink arrows indicate MFBs and MFs, respectively. Bars = 1 cm.

1

Comparison and phylogenetic analysis of *JcAP1* and other *AP1* genes.

(A) Sequence alignment of the JcAP1, AtAP1, PtAP1, and VvAP1 amino acid sequences. Similar amino acid residues are shaded in black. Dots denote gaps. Each colored line under the alignment indicates a different domain of an AP1 homolog. "M-box" indicates the highly conserved MADS-box domain; "I domain" indicates the intervening domain; "K-box" indicates the conserved keratin-like domain; "C domain" indicates the C-terminal domain; and “euAP1 motif” indicates the euAP1 motif. (B) Phylogenetic analysis of AP1 homologs from different plant species: AtAP1, Arabidopsis thaliana *AP1 (NP_177074); BpAP1, Betula platyphylla* AP1 (AFV92462); BrAP1, Brassica rapa *AP1 (XP_009105460.1); DlAP1-1, Dimocarpus longan* AP1-1 (AEZ63951); DlAP1-2, Dimocarpus longan *AP1-2 (AGC13077); JcAP1, Jatropha* curcas *AP1 (KR013222); PtAP1-1, Populus trichocarpa* AP1-1 (XP_002311353); PtAP1-2, Populus trichocarpa *AP1-2 (XP_002316076); RcAP1, Ricinus communis AP1 (XP_002514623); CaAP1, Coffea arabica* AP1 (AHW58038); MdAP1, Malus domestica *AP1 (ACD69426);* SiAP1, Sesamum indicum *AP1 (AIS82596); VvAP1, Vitis vinifera* AP1 (NP_001268210); PgAP1, Panax ginseng *AP1 (BAK20019); LjAP1, Lotus japonicus* AP1 (AAX13296); VuAP1, Vigna unguiculata *AP1 (BAJ22385); CsAP1, Camelina sativa* AP1 (XP_010415539); TcAP1, Theobroma cacao *AP1* (XP_007045796); PvAP1, Plukenetia volubilis *AP1* (Ku942379); MeAP1, Manihot esculenta *AP1(_029935m http://treetfdb.bmep.riken.jp); PsAP1, Pisum sativum* AP1 (AAL66379); GmAP1, Glycine max *AP1 (XP_003531957); ZjAP1, Ziziphus jujube* AP1 (ACG70964); AtCAL, Arabidopsis thaliana *CAL (NP_564243); BrCAL, Brassica rapa* CAL (XP_009109914); CsCAL, Camelina sativa *CAL (XP_010477869); AtFUL, Arabidopsis thaliana* FUL (NP_568929); CaFUL, Coffea arabica *FUL (AHW58040); JcFUL, Jatropha* curcas *FUL (KDP31379); PtFUL, Populus trichocarpa* FUL (ABK92820); VvFUL, Vitis vinifera *(XP_002263017); RcFUL, Ricinus communis* FUL (KDP31379); BrFUL, Brassica rapa *FUL (XP_009130138); CsFUL, Camelina sativa* FUL (XP_010443902); TcFUL, Theobroma cacao[i] FUL (XP_007037634); The phylogeny of these

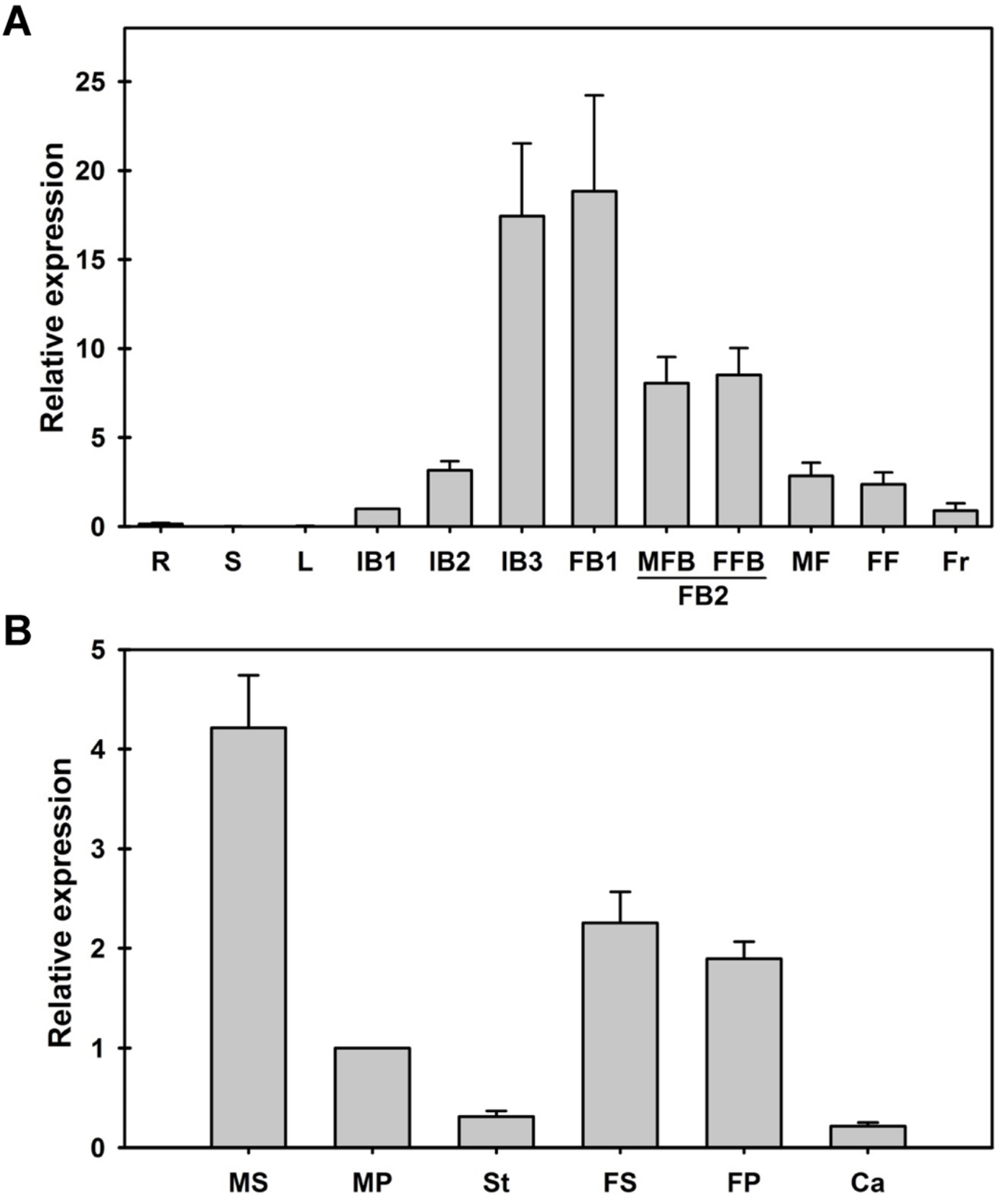
AP1 homologs was determined based on their amino acid sequences using MEGA5 and the neighbor-joining method. Bootstrap values were obtained using 1000 bootstrap replicates.



2

Expression of *JcAP1* in various adult Jatropha organs.

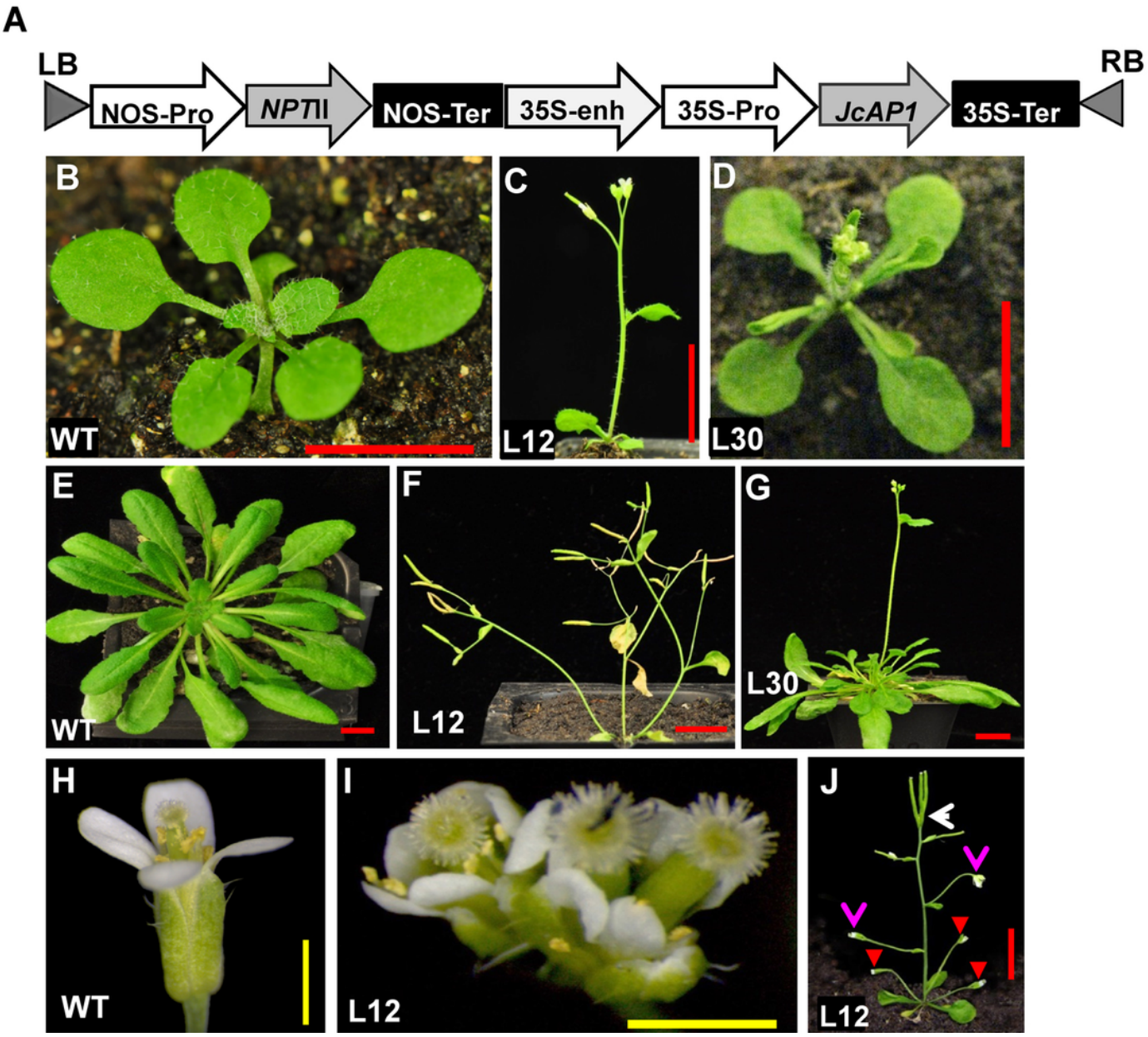
(A) The expression level of *JcAP1* in the roots, stems, leaves, inflorescence buds, flower buds, flowers and fruits. (B) The expression level of *JcAP1* in different whorls of male and female flowers. The qRT-PCR results were obtained from two independent biological replicates and three technical replicates for each sample. The error bars represent the standard deviation. R: roots; S: stems; L: mature leaves; IB1: inflorescence bud stage 1 (0–5 days, inflorescence buds are visible); IB2: inflorescence bud stage 2 (1 week after IB1); IB3: inflorescence bud stage 3 (1 week after IB2); FB1: flower bud stage 1 (1 week after IB3); FB2: flower bud stage 2 (1 week after FB1, the male flower bud (MFB) and female flower bud (FFB) are identifiable); MF: male flower (1 week after MFB); FF: female flower (1 week after FFB). Fruits (Fr) were harvested 15 days after fertilization. Male sepals (MS), male petals (MP), stamens (St), female sepals (FS), female petals (FP), and carpels (Ca) were harvested 1 or 2 days before the male and female flowers bloomed. The levels of the detected amplicons were normalized using the amplified product of *JcACTIN1*. The mRNA levels in the IB1 and male petal tissues were used as standards, with a set value of 1.


A
Relative expression
25
20
15
10
5
0
R
S
L
IB1
IB2
IB3
FB1
MFB
FFB
FB2
MF
FF
Fr
B
Relative expression
5
4
3
2
1
0
MS
MP
St
FS
FP
Ca

3

Ectopic expression of *JcAP1* results in early flowering and abnormal flowers in transgenic Arabidopsis.

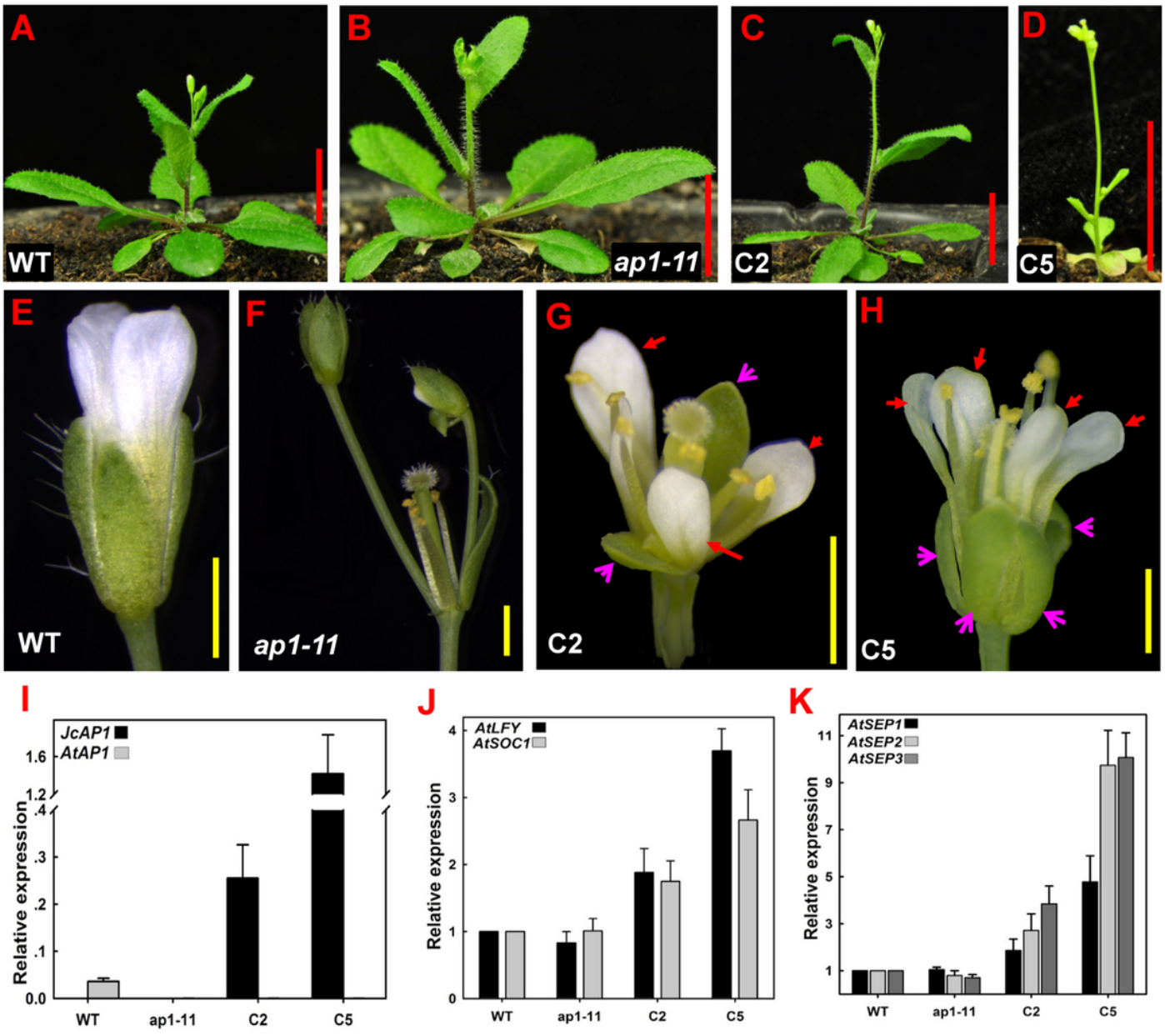
(A) Schematic diagram of the T-DNA region of the binary plasmid used in this study. The two-way arrows indicate the sequences of the non-T-DNA region. NOS-Pro, nopaline synthase promoter; 35S-Pro, CaMV 35S promoter; 35S-Enh, CaMV 35S enhancer; NOS-Ter, nopaline synthase terminator; CaMV 35S-Ter, 35S terminator; RB, right border of the T-DNA region; and LB, left border of the T-DNA region. (B–D) 15 days seedlings grew under LD conditions. (E–G) 60 days seedlings grew under SD conditions. (H) A normal flower of a wild-type plant. (I) Abnormal flowers of 35S:*JcAP1* transgenic plants from L12. (J) A solitary flower appeared at the rosette and cauline leaf axils in the transgenic plants. Red arrows indicate the solitary rosette flowers; pink arrows indicate that primary shoots immediately terminated with the formation of asolitaryflower; the white arrow indicates fruit formation of the abnormal terminal flowers. (B, E, and H) WT; (C, F, I, and J) transgenic L12; (D and G) transgenic Arabidopsis L30. Red bars = 1 cm, yellow bars = 1 mm.


A
LB
NOS-Pro
NPTII
NOS-Ter
35S-enh
35S-Pro
JcAP1
35S-Ter
RB
B
WT
C
L12
D
L30
E
WT
F
L12
G
L30
H
WT
I
L12
J
L12

4

35S:*JcAP1* transgenic Arabidopsis recovers the phenotype of the *ap1-11* mutant and causes early flowering.

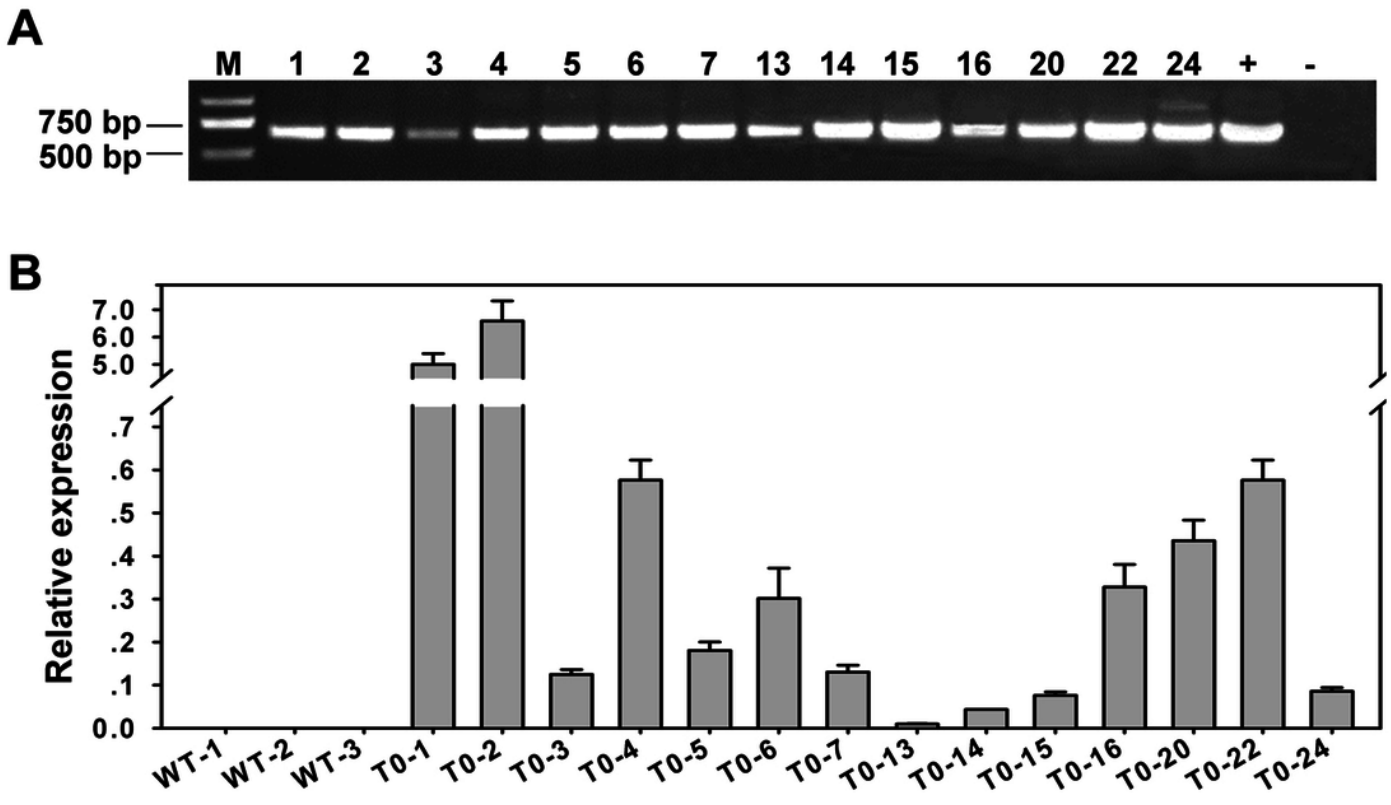
(A–C) WT, *ap1-11*, and 35S:*JcAP1* complementary *ap1-11* plant line 2 (C2) growth under LD conditions at 30 days after germination. (D) 35S:*JcAP1* complementary *ap1-11* plant line 5 (C5) growth under LD conditions at 15 days after germination. (E–H) WT (E), *ap1-11* (F), and 35S:*JcAP1* florets in *ap1-11* rescued sepals and petals (G and H). The red arrow indicates that the petals appeared in complementary transgenic plants, and the pink arrow indicates that the sepals appeared in complementary transgenic plants. (I–K) qRT-PCR analysis of *JcAP1* and other flowering-related genes including Arabidopsis *APETALA1 (AtAP1*), *LEAFY (AtLFY)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (AtSOC1*), *SEPALLATA* 1*, 2* and 3 *(AtSEP1*, AtSEP2*, and AtSEP3*) in WT, ap1-11 and transgenic Arabidopsis (C2, C5). The levels of the detected amplicons were normalized using the amplified products of *AtACTIN2.* The mRNA level in WT was set as the standard, with a value of 1. Red bars = 1 cm, yellow bars = 1 mm.


A
WT
B
ap1-11
C
C2
D
C5
E
WT
F
ap1-11
G
C2
H
C5
I
JcAP1
AtAP1
Relative expression
WT
ap1-11
C2
C5
J
AtLFY
AtSOC1
Relative expression
WT
ap1-11
C2
C5
K
AtSEP1
AtSEP2
AtSEP3
Relative expression
WT
ap1-11
C2
C5

5

Analysis of the transgenic Jatropha plants.

(A) Amplification of the 600-bp fragment containing partial sequences of the 35S promoter and *JcAP1* cDNA. Lanes: M, Trans 2 Kb DNA ladder; +, positive control (plasmid); -, negative control (wild type); and 14 regenerated transgenic Jatropha lines. (B) Quantitative RT-PCR analysis of 3 WT and 14 independent transgenic plants (L1, L2, L3, L4, L5, L6, L7, L13, L14, L15, L16, L20, L22, L24). Two transgenic plants, L2 and L20, showing high and intermediate expression levels, respectively, were chosen for further analysis. RNA was extracted from young leaves of 2-month-old plantlets. The transcript levels were normalized using the *JcACTIN1* gene as a reference.



6

The flowering time of 35S:*JcAP1* transgenic Jatropha in the field.

(A) WT plant grown in a pot for 2 months, at the vegetative growth stage; (B) 35S:*JcAP1* transgenic Jatropha grown in a pot for 2 months, at the vegetative growth stage. (C) WT plants grown in the field for 4 months, at the anthesis stage; (D) The inflorescence of wild Jatropha in the field. (E, G) 35S:*JcAP1* transgenic Jatropha L2 and L20 plants grown in the field for 4 months, at the anthesis stage; (F, H) The inflorescence of transgenic Jatropha in the field. (I) The flowers of WT and 35S:*JcAP1* transgenic Jatropha L2 and L20 plants. Red bars = 1 cm, white bars = 10 cm, yellow bars = 50 cm.