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REGULAR PAPER

A promoter analysis of *MOTHER OF FT AND TFL1 1* (*JcMFT1*), a seed-preferential gene from the biofuel plant *Jatropha curcas*

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Abstract *MOTHER OF FT AND TFL1* (*MFT*)-like genes belong to the phosphatidylethanoamine-binding protein (PEBP) gene family in plants. In contrast to their homologs *FLOWERING LOCUS T* (*FT*)-like and *TERMINAL FLOWER 1* (*TFL1*)-like genes, which are involved in the regulation of the flowering time pathway, *MFT*-like genes function mainly during seed development and germination. In this study, a full-length cDNA of the *MFT*-like gene *JcMFT1* from the biodiesel plant *Jatropha curcas* (L.) was isolated and found to be highly expressed in seeds. The promoter of *JcMFT1* was cloned and characterized in transgenic *Arabidopsis*. A histochemical β-glucuronidase (GUS) assay indicated that the *JcMFT1* promoter was predominantly expressed in both embryos and endosperms of transgenic *Arabidopsis* seeds. Fluorometric GUS analysis revealed that the *JcMFT1* promoter was highly active at the mid to late stages of seed development. After seed germination, the *JcMFT1* promoter activity decreased gradually. In addition, both the *JcMFT1* expression in germinating *Jatropha* embryos and its promoter activity in germinating *Arabidopsis* embryos were induced by abscisic acid (ABA), possibly due to two ABA-responsive elements, a G-box and an RY repeat, in the *JcMFT1* promoter region. These results show that the *JcMFT1* promoter is seed-preferential and can be used to control transgene expression in the seeds of *Jatropha* and other transgenic plants.

Keywords ABA · *MOTHER OF FT AND TFL1* (*MFT*) · Physic nut · Promoter · Seed

Z.-F. Xu is a non-member of the Botanical Society of Japan.

Y.-B. Tao · L.-L. He · J. Ni · Z.-F. Xu (✉)
Key Laboratory of Tropical Plant Resource and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Yunnan 666303, China
e-mail: zfxu@xtbg.ac.cn

Y.-B. Tao
e-mail: taoyanbin0707@gmail.com

L.-L. He
e-mail: liangl.he08@gmail.com

J. Ni
e-mail: nijun.sun@gmail.com

Y.-B. Tao
University of Chinese Academy of Sciences, Beijing 100049, China

L. Luo
Key Laboratory of Gene Engineering of Ministry of Education and State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, Guangdong, China
e-mail: lilian1986.2008@gmail.com

L.-L. He · J. Ni
School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

Introduction

Physic nut (*Jatropha curcas*, hereafter referred to as *Jatropha*), a member of the Euphorbiaceae family, is a small tree or large shrub that originated in Mexico and Central America (Heller 1996). *Jatropha* seeds contain approximately 30–40 % oil, which is a promising level for biodiesel production (Abdulla et al. 2011; Chakrabarti and Prasad 2012; Fairless 2007; King et al. 2009; Makkar and Becker 2009) and bio-jet fuel (Li et al. 2010). However, to optimize its use, there are a number of *Jatropha* traits that must be improved, such as seed yield, freezing tolerance,

pest and disease resistance, oil content, seed toxicity, and synchronous flowering/fruiting (Abhilash et al. 2011; Achten et al. 2010; Divakara et al. 2010; Pan and Xu 2011; Sanderson 2009). Both conventional and molecular breeding approaches are used for *Jatropha* improvement (Argollo Marques et al. 2013; Chikara et al. 2013; Gressel 2008; Sujatha et al. 2008; Yue et al. 2013). The transgenic approach has the potential to significantly improve *Jatropha* agronomic and economic traits and requires various constitutive, tissue-specific or inducible promoters (Jha et al. 2013; Kumar et al. 2013; Pan et al. 2010; Qu et al. 2012; Tsuchimoto et al. 2012).

The PEBP gene family is evolutionarily conserved in prokaryotes (Serre et al. 2001) and eukaryotes (Banfield et al. 1998; Banfield and Brady 2000). In plants, the PEBP gene family is divided into three subfamilies: the *FLOWRING LOCUS T* (*FT*)-like clade, the *TEMINAL FLOWER1* (*TFL1*)-like clade and the *MOTHER OF FT AND TFL1* (*MFT*)-like clade (Chardon and Damerval 2005; Hedman et al. 2009). *FT*-like and *TFL1*-like genes act on the transition from the vegetative to the reproductive phase in plants (Ahn et al. 2006; Kobayashi et al. 1999). *MFT*-like genes are involved in the development of the reproductive organs in species from mosses to angiosperms, whereas the occurrence of the *FT-like* and *TFL1*-like genes is associated with the evolution of seed plants, and therefore, the *MFT*-like clade is likely to be ancestral to the *FT*-like and *TFL1*-like clades (Chardon and Damerval 2005; Hedman et al. 2009; Karlgren et al. 2011).

Most of the *MFT*-like genes have been reported to express predominantly in seeds. In the gymnosperm *Picea abies*, *PaMFT1* and *PaMFT2* were detected at a high expression level in zygotic embryos (Karlgren et al. 2011). In angiosperms, *MFT* was initially identified as a redundant floral inducer with *FT*-like genes in *Arabidopsis* (Yoo et al. 2004). However, according to the expression pattern and transgenic analyses in subsequent studies, it appears that *MFT*-like genes play a greater role in the seed development pathway than in the flowering time pathway (Chardon and Damerval 2005; Kikuchi et al. 2009; Nakamura et al. 2011). In fact, the *Arabidopsis MFT* was highly expressed in seeds (Danilevskaya et al. 2008; Xi et al. 2010). *PnFTL4* from Lombardy poplar (*Populus nigra*), an ortholog of *Arabidopsis MFT*, was also expressed mainly in seeds (Igasaki et al. 2008). A high expression level of Satsuma mandarin (*Citrus unshiu*) *CuMFT1* was detected in mature seeds, and an analysis in *Arabidopsis* showed strong promoter activity in the seeds of this species (Nishikawa et al. 2008). *Arabidopsis MFT* is also a negative regulator of ABA signaling that promotes embryonic growth (Xi et al. 2010). By contrast, in wheat (*Triticum aestivum*), *TaMFT* expression suppresses seed germination (Nakamura et al. 2011).

In the present study, we isolated *JcMFT1*, an ortholog of *Arabidopsis MFT* from *Jatropha*, and found that it is also expressed predominantly in seeds. Using the β-glucuronidase (GUS) reporter gene (Jefferson et al. 1987), we found that the *JcMFT1* promoter is highly activated in transgenic *Arabidopsis* seeds and is inducible by ABA in germinating seeds, which is consistent with the presence of ABA-responsive elements in the *JcMFT1* promoter. Thus, the *JcMFT1* promoter could be utilized as a seed-preferential promoter for plant genetic engineering and the functional analysis of genes involved in seed development in *Jatropha* and other plants.

Materials and methods

Plant materials

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

Cloning and quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) analysis of *JcMFT1* in *Jatropha*

A partial cDNA sequence of the *Arabidopsis MFT* homolog was identified in our *Jatropha* embryo EST library (Chen et al. 2011). To obtain the full-length *JcMFT1* cDNA, 3′ rapid amplification of cDNA ends (RACE) was performed with total RNA from embryos following the SMART™ RACE cDNA Amplification Kit User Manual (Clontech). Total RNA from embryos was isolated using the silica particle extraction method (Ding et al. 2008). A gene-specific primer, GSP1, was designed for 3′ RACE, based on the partial *JcMFT1* cDNA sequence, together with the adaptor primers AP1 and AP2. The PCR products were cloned into the pGEM-T Easy vector (Promega) for sequencing. The full-length *MFT* cDNA was then amplified from embryo cDNA by PCR using the primers ZF533 (forward) and ZF579 (reverse).

The expression levels of *JcMFT1* were measured in various organs of *Jatropha* using qRT–PCR. Total RNA was isolated (Ding et al. 2008) and reverse transcribed using the PrimeScript® RT reagent Kit with the gDNA Eraser (TAKARA). qRT–PCR was performed using SYBR® Premix Ex Taq™ II (TAKARA) with a Roche 480 Real-time PCR detection system (Roche Diagnostics). The expression of *JcGAPDH* was used as an internal control to normalize all gene expression data obtained by qRT–PCR. The primers used in gene cloning and qRT–PCR are listed in Table 1.

Table 1 The sequences of the primers used in this study

Name	Sequence (from 5′ to 3′)	Feature
AP1	GTAATACGACTCACTATAGGGC	Adaptor primer for genome walking and 3′ RACE
AP2	ACTATAGGGCACGCGTGGT	Adaptor primer for genome walking and 3′ RACE
GSP1	TGTCTCTCTTCTTCTCGTTTTAATGGCGG	*JcMFT1* gene-specific primer for 3′ RACE
GSP2	GCCGCCATTAAAACGAGAAGAAGAGA	*JcMFT1* gene-specific primer for genome walking
GSP3	GCCAGAAATGGTAAGTTTAGGAGGGT	*JcMFT1* gene-specific primer for genome walking
GSP4	TACACTCTTTGCTTACTTCAATAGCGA	*JcMFT1* gene-specific primer for genome walking
XA79	CTCTGTGTCTCTCTTCTTCTCGTTTTA	*JcMFT1* gene primer for qRT–PCR
XA80	CCAGAAATGGTAAGTTTAGGAGGGT	*JcMFT1* gene primer for qRT–PCR
XT95	GCTGCTAAGGCTGTTGGGAA	*JcGAPDH* gene primer for qRT–PCR
XT96	GACATAGCCCAATATTCCCTTCAG	*JcGAPDH* gene primer for qRT–PCR
ZF533	CGGGATCCTTCTTCTCGTTTTAATGG	For cloning the full-length cDNA of *JcMFT1*
ZF579	GAGAGCTCGAGAAGGACTTAGCGCCT	For cloning the full-length cDNA of *JcMFT1*
XT378	TGCTCTAGACCTATGAATGTTAGTTTGA	For construction of *JcMFT1:GUS*. The added *Xba*I site was underlined
XT394	CGCGGATCCTAAAACGAGAAGAAGAGAG	For construction of *JcMFT1:GUS*. The added *Bam*HI site was underlined

Cloning of the 5′ flanking region of *JcMFT1*

The 5′ flanking region upstream of the translation start codon of *JcMFT1* was isolated from *Jatropha* genomic DNA by genome walking according to the GenomeWalker™ Kit Universal User Manual (Clontech). For nested PCR, the *JcMFT1* gene-specific primers GSP2, GSP3, and GSP4 and the adaptor primers AP1 and AP2 were used. The *JcMFT1* promoter was amplified by PCR using primers XT378 (forward) and XT394 (reverse) carrying the *Xba*I and *Bam*HI restriction sites, respectively, and the resulting fragments were cloned into the pGEM-T Easy vector for sequencing. The putative *cis*-acting elements of the *JcMFT1* promoter were analyzed using the PLACE database (Higo et al. 1999). The primers used in genome walking are listed in Table 1.

Construction of the promoter-GUS fusion and *Arabidopsis* transformation

To generate the *JcMFT1 promoter-GUS* plasmid, a 1.5 kb *Xba*I–*Bam*HI *JcMFT1* promoter fragment of the above mentioned pGEM-T Easy vector was subcloned into the *Xba*I–*Bam*HI sites of pBI101 (Jefferson et al. 1987). The resulting construct, designated *JcMFT1pro:GUS* (Fig. 3b), was transferred into *A. tumefaciens* EHA105 by electroporation (GenePulser Xcell, Bio-Rad), and the resulting *A. tumefaciens* was used to transform *Arabidopsis* by the floral dip method (Clough and Bent 1998).

ABA treatment

Mature *Jatropha* seeds were first sterilized with 75 % (v/v) ethanol for 30 s and rinsed three times with sterile water, and then sterilized with 10 % (v/v) sodium hypochlorite for 20 min and rinsed five times with sterile water. Embryos dissected from seeds were sown on MS medium supplemented with 0, 50, 100, 200, and 400 μM ABA and collected after being cultured at 28 °C under a 14 h light/10 h dark photoperiod for 24 h. Then the *JcMFT1* expression was examined by qRT–PCR in these embryos.

Homozygous T2 seeds from transgenic lines 2 and 4 were used for ABA treatment. The mature seeds were sterilized with 20 % commercial Clorox bleach for 15 min and washed twice with sterile water. The sterile seeds were sown on 1/2 MS medium supplemented with 10 μM ABA (Xi et al. 2010). The control medium contained an equal amount of mock solution. After stratification at 4 °C for two days, the seeds were germinated at 22 °C under a 16 h light/8 h dark photoperiod. For histochemical GUS staining, mock-treated or ABA-treated germinating seeds at the same developmental stage were collected 14 or 24 h after stratification, respectively. The experiment was repeated three times.

Histochemical and fluorometric GUS assays

For histochemical GUS staining, the developing seedlings and various organs of the T2 transgenic *Arabidopsis* plants

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 and then cleared with 70 % ethanol (Jefferson et al. 1987). The samples were examined by stereomicroscopy (Leica M80).

To examine the activity of the *JcMFT1* promoter during seed development, T3 transgenic seeds at various developmental stages were collected. A fluorometric GUS assay was performed following the protocol described by Jefferson et al. (1987) with the addition of 2 mM MUG to the reaction buffer. The fluorescence was examined using a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation). The protein concentrations in the plant extracts were measured using the Bradford method (1976).

Results

The isolation of *Jatropha JcMFT1* and its phylogenetic analysis

Using a partial cDNA sequence derived from a *Jatropha* embryo EST library (Chen et al. 2011), we isolated a full-length cDNA ortholog in *Jatropha* for *Arabidopsis MFT*, and, the clone was designated *JcMFT1* (GenBank accession No. KC874668). *JcMFT1* encodes 172 amino acids. As shown in Fig. 1a, the protein sequences of the MFT-like gene family members are highly conserved among various species. The key residue Trp83, which contributes to MFT function in *Citrus unshiu* and *Arabidopsis* (Hanzawa et al. 2005; Nishikawa et al. 2008), was identified in JcMFT1 (Fig. 1a, indicated by an asterisk). We also found another *MFT*-like gene in the updated Jatropha Genome Database (Release 4.5, http://www.kazusa.or.jp/jatropha/), and here designated *JcMFT2* (accession No. KF944352). We performed phylogenetic analysis to determine the evolutionary relationship between JcMFT and other members of the PEBP family. The results showed that JcMFT1 is most closely related to CuMFT and AtMFT (Fig. 1b). This finding suggested that *JcMFT1* might share a similar function with *CuMFT* and *AtMFT*, both of which are predominantly expressed in seeds (Danilevskaya et al. 2008; Nishikawa et al. 2008). In contrast, JcMFT2 was closely related to grapevine VvMFT and tomato SP2I. *SP2I* was expressed in all tested organs and developmental stages (Carmel-Goren et al. 2003), and *VvMFT* showed a low level of expression in seeds (Carmona et al. 2007).

The expression pattern of *JcMFT1* in *Jatropha*

To explore the expression pattern of *JcMFT1* in *Jatropha*, total RNA extracted from various tissues was analyzed by qRT–PCR. The result revealed that *JcMFT1* expression in mature seeds (56 days after pollination, DAP) was much higher than in various tissues of adult plants (Fig. 2a). During seed development, as shown in Fig. 2b, *JcMFT1* started to be expressed at seven days after pollination (DAP), then showed undetectable expression level from 14 to 21 DAP. At 28 DAP, at which stage the seed volume increases rapidly (Fig. 2d), the expression level of *JcMFT1* significantly increased, and peaked at 42 DAP (Fig. 2b), when the seed coat was completely black (Fig. 2d). Subsequently, *JcMFT1* expression decreased rapidly until the seeds were desiccated at 56 DAP (mature seeds). These results indicate that *JcMFT1* is mainly expressed at the mid to late stages of seed development.

To determine whether *JcMFT1* is also involved in the regulation of seed germination, further analysis of *JcMFT1* expression was performed in germinating seeds. The results showed that the expression level of *JcMFT1* decreased gradually along with seed germination, and *JcMFT1* expression was detected in embryos and endosperms up to 48 h after sowing (HAS) in soil. Up to 24 HAS, a much higher expression level of *JcMFT1* was found in embryos as compared to endosperms. After 24 HAS, *JcMFT1* expression was very low in both embryos and endosperms (Fig. 2c). In comparison with its expression in seeds, *JcMFT1* expression in seedlings was very weak (Fig. 2c).

Isolation and sequence analysis of the *JcMFT1* promoter

The 1.5 kb *JcMFT1* promoter fragment (GenBank accession No. KC874669) was isolated from *Jatropha* genomic DNA by genome walking (Siebert et al. 1995). An analysis of putative *cis*-acting elements in this promoter region was performed using the PLACE database (Higo et al. 1999). The putative plant regulatory elements are shown in Fig. 3a, and the sequences on both strands were considered. The *JcMFT1* promoter contains multiple elements involved in seed-specific transcriptional regulation, including the AACA core (AACAAAC) (Suzuki et al. 1998), the $(CA)_n$ element (CNAACAC) (Ellerström et al. 1996), the DOF core (AAAG) (Yanagisawa and Schmidt 1999), the E box (CANNTG) (Kawagoe and Murai 1992; Stålberg et al. 1996), the prolamin box (TGCAAAG) (Vicente-Carbajosa et al. 1997), the RY repeat (CATGCA) (Lelievre et al. 1992) and the SEF1, SEF3, SEF4 motifs (1, ATATTTAWW; 3, AACCCA; and 4, RTTTTTR) (Lessard et al. 1991). These elements are believed to confer promoter activity in seeds. The RY repeat (CATGCA) and the G box (CACGTG) (Menkens et al. 1995) found in this promoter are ABA-responsive elements (ABRE) (Ezcurra et al. 1999; Sibéril et al. 2001), suggesting that the activity of the *JcMFT1* promoter may be influenced by ABA.

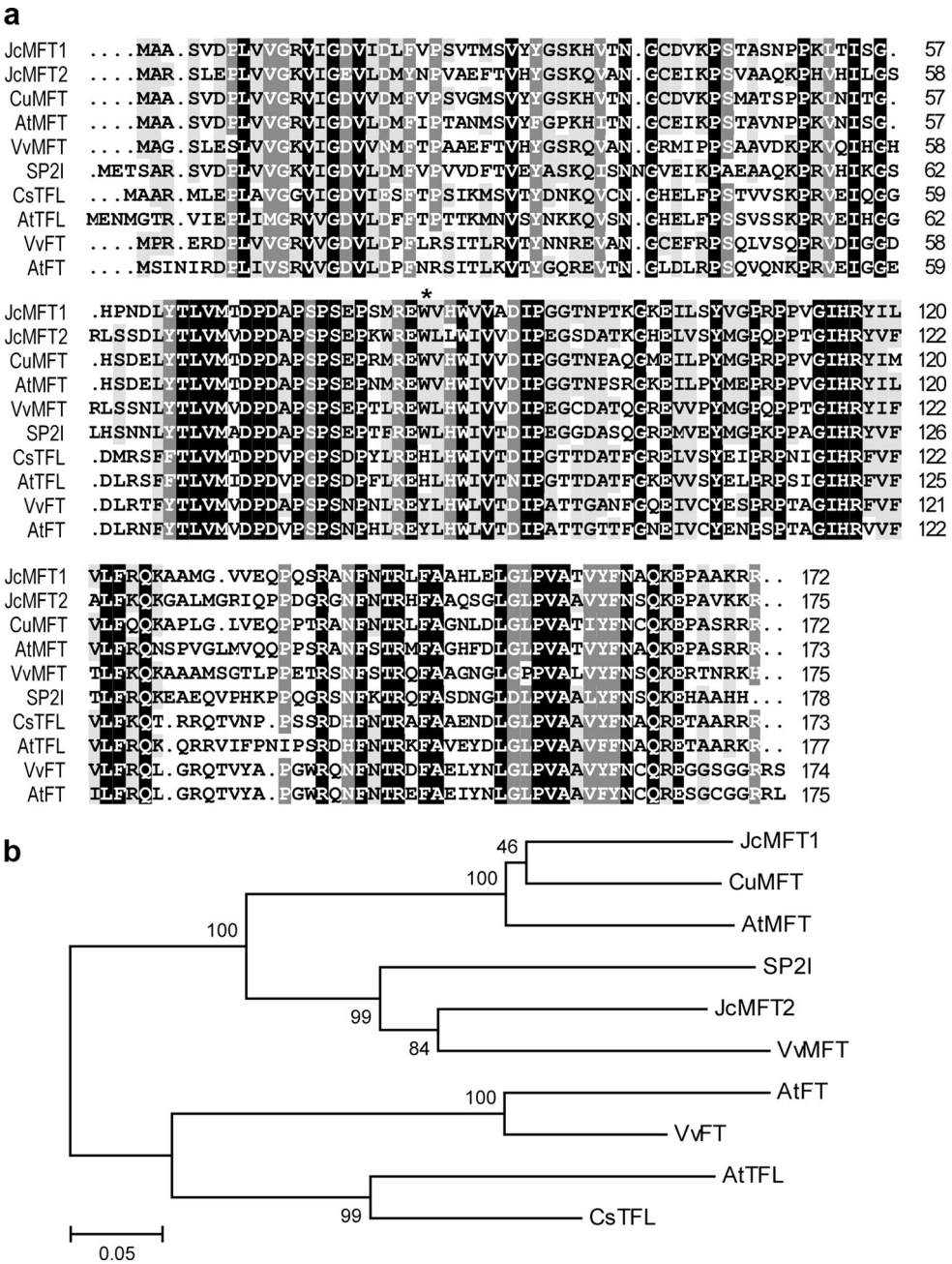

Fig. 1 A comparison of JcMFTs with their homologs. **a** An alignment of deduced amino acid sequences between different MFT-related proteins. JcMFT1, from *J. curcas*, accession No. KC874668; JcMFT2, from *J. curcas*, accession No. KF944352; CuMFT, from *Citrus unshiu*, accession No. AB304142; AtMFT, from *A. thaliana*, accession No. NM101672; VvMFT, from *Vitis vinifera*, accession No. DQ871594; SP2I, from *Lycopersicon esculentum* (tomato) accession No. AA031791; CsTFL, from *C. sinensis*, accession No. AY344244; AtTFL, from *A. thaliana*, accession No. NM_120465; VvFT, from *V. vinifera*, accession No. EF157728; and AtFt, from *A. thaliana*, accession No. NM_105222. Identically and partially conserved amino acid sequences are shown in black and gray, respectively. The asterisk indicates the key residue that contributes to MFT function. **b** A phylogenetic tree of JcMFT1 and other homologs. The tree was constructed using MEGA 5.0 software and the neighbor-joining (N-J) method. The N-J unrooted dendrogram was generated from an alignment of the deduced amino acids with the ClustalW program. One thousand replicates were used for the Bootstrap test. The *scale bar* indicates the average number of substitutions per site

The expression profile of a *JcMFT1* promoter-GUS fusion in transgenic *Arabidopsis*

A *JcMFT1 promoter-GUS* fusion (Fig. 3b) was constructed for transformation and analysis in *Arabidopsis*. A histochemical GUS assay was performed on four independent transgenic lines, all of which showed the same GUS expression pattern. We analyzed roots, leaves, inflorescence stems, flowers, and green siliques bearing seeds from plants at the reproductive stage, and we found that GUS staining was only observed in the seeds (Fig. 4a–e). However, in just-germinated seeds in which the radicle tip had barely appeared (Xi et al. 2010), *GUS* was highly expressed in whole embryos with the strongest activity in the tips of the radicles (Fig. 4g). Next, the GUS activity was analyzed in seedlings at 1, 2, 4, 6, and 9 days after germination (DAG). GUS staining was observed in whole seedlings with extraordinary intensity in the cotyledons at 1 DAG (Fig. 4h). From 2 DAG onward, GUS activity in the hypocotyls and cotyledons decreased progressively, and no visible activity was detected in the roots (Fig. 4i–l). At 9 DAG, weak GUS staining was only observed in the cotyledons (Fig. 4l). The true leaves appearing at 4 DAG did not show any visible activity (Fig. 4j–l). The results

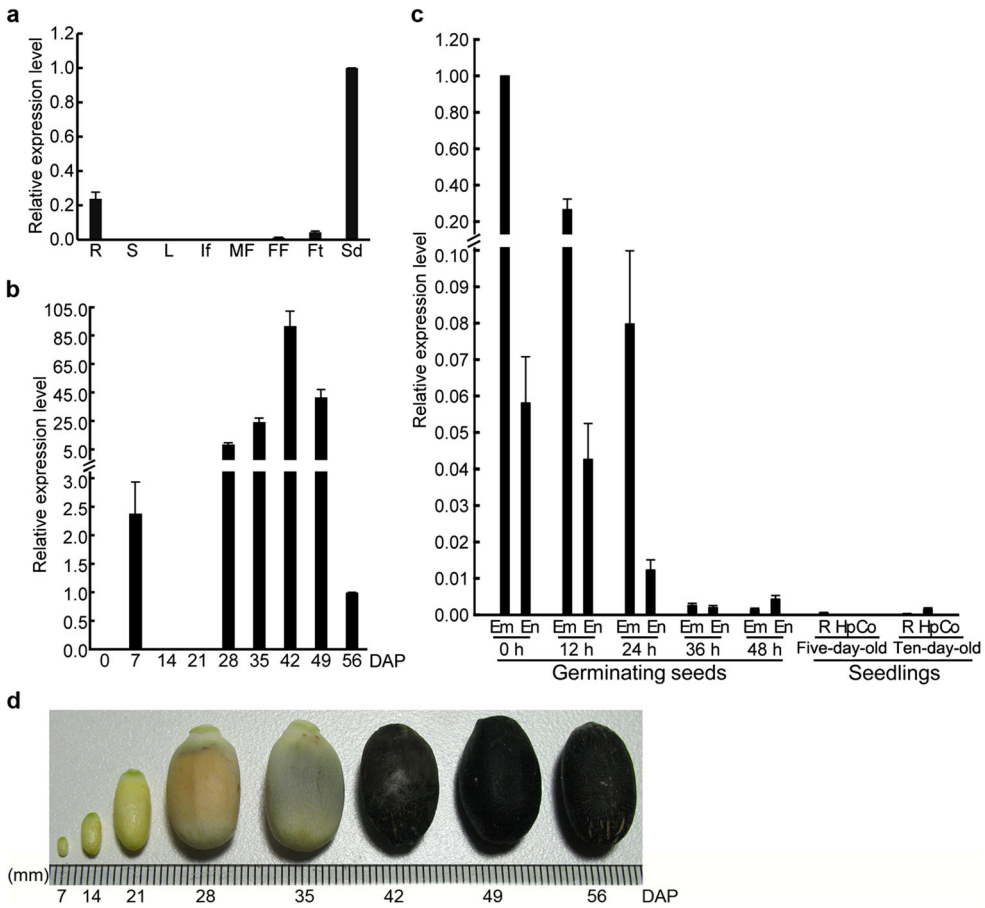

Fig. 2 The expression pattern of *JcMFT1* in *Jatropha*. **a** *JcMFT1* expression in various organs of adult plants. The relative mRNA level of mature seeds was set as the standard value of 1. *R* roots, *S* stems, *L* leaves, *If* inflorescence stems, *MF* male flowers, *FF* female flowers, *Ft* fruits, *Sd* mature seeds at 56 days after pollination (DAP). **b** *JcMFT1* expression during seed development. The relative mRNA level of mature seeds (56 DAP) was set as the standard value of 1. **c** *JcMFT1* expression during seed germination and seedling development. The relative mRNA level of endosperm at 0 h was set as the standard value of 1. *Em* embryos, *En* endosperms, *R* roots, *Hp* hypocotyls, *Ct* cotyledons. **d** The appearance of seeds from 7 to 56 DAP. Equivalent qRT–PCR results were obtained from duplicate biological replicates. The *error bars* denote the SD from triplicate technical replicates. The values were normalized using the expression of the reference gene *JcGAPDH*

from this analysis indicated that the activity of the *JcMFT1* promoter was confined to seeds and cotyledons at early developmental stages.

Activity of the *JcMFT1* promoter in the seed development of transgenic *Arabidopsis*

Although the *JcMFT1* promoter was highly active in seeds, initially, we were not able to detect visible GUS activity at the early developmental stages of seeds. A further analysis of promoter activity was performed during seed development. Seeds from two homozygous transgenic lines were examined with a fluorometric GUS assay. The result (Fig. 5) showed that the GUS activity increased markedly from 9 to 15 days after flowering (DAF), the time period when the developing seeds transition from the cotyledon stage to the mature stage (Le et al. 2010). The siliques began to turn yellow at 15 DAF, while the seeds were still green. When the seeds turned completely yellow at 21 DAF, the GUS activity was reduced to one-third of that at 15 DAF (Fig. 5). Although the GUS activity in transgenic line 4 was higher than that in line 2, the expression pattern of *GUS* during seed development of the two lines was similar (Fig. 5). This result demonstrates that the *JcMFT1* promoter is active at the mid to late stages of seed development, which is consistent with *JcMFT1* expression in *Jatropha* (Fig. 2). Furthermore, we dissected the GUS-stained seeds at the mid to late developmental stages, and found that the GUS expression was persistent in both embryos and endosperms from the linear cotyledon stage (8 DAP) to the post mature stage (18 DAP), but no expression was detected in seed coats (Fig. 6).

Fig. 3 The *JcMFT1* promoter-reporter gene construct. **a** The nucleotide sequence of the *JcMFT1* promoter. The A of the start codon ATG (*bold and boxed*) is numbered as +1. The putative regulatory elements on both strands are shown in *bold and underlined*. **b** A schematic structure of the T-DNA region of the *JcMFT1-GUS* binary vector used for *Arabidopsis* transformation

The *JcMFT1* promoter is induced by ABA during *Arabidopsis* seed germination

Because the *JcMFT1* promoter contains several ABREs (Fig. 3a) and because *JcMFT1* expression declined during seed germination (Fig. 2c) when ABA levels are low (Kucera et al. 2005), we speculated that this promoter might be regulated by ABA. To test this hypothesis, we first examined whether *JcMFT1* expression in *Jatropha* was affected by ABA treatment. We found that *JcMFT1* expression was up-regulated by exogenous ABA in germinating *Jatropha* embryos, and the highest expression level was observed at 100 μM of ABA (Fig. 7a). Next, we tested the *JcMFT1* promoter activity by treating transgenic *Arabidopsis* seeds with 10 μM ABA. Compared with mock treatment (Fig. 7b), the GUS staining was observed in whole embryos when exogenous ABA was applied (Fig. 7c). Apparent ABA induction of *JcMFT1* promoter activity was also found in the fluorometric assay of GUS activity in two transgenic plant lines (Fig. 7d). These

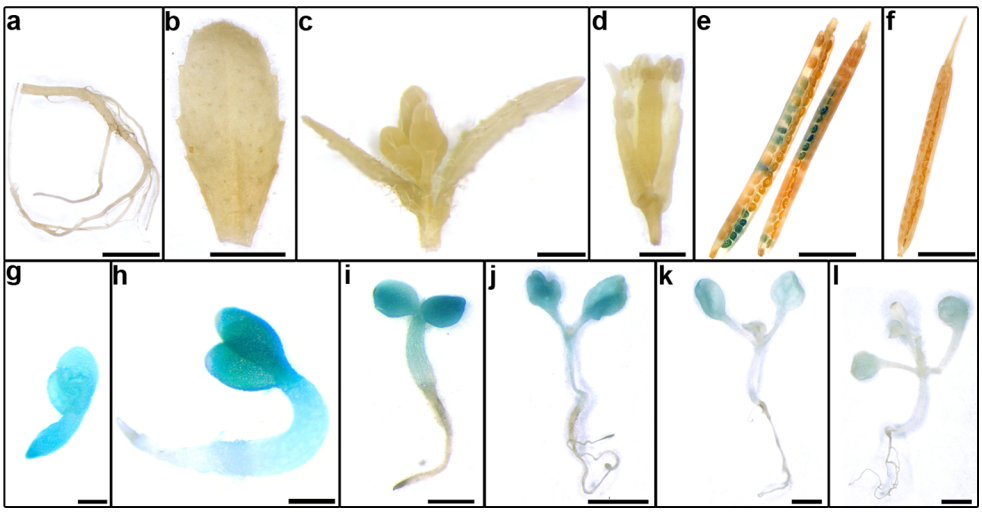

Fig. 4 Histochemical GUS staining of transgenic *Arabidopsis* carrying a *JcMFT1 promoter-GUS* fusion. Various organs of adult plants: **a** root, **b** leaf, **c** inflorescence stem, **d** flower, **e** siliques bearing seeds, **f** wide-type silique bearing seeds, **g** an embryo from a just-germinated seed. Different stages of seedlings: **h** one day after germination (DAG), **i** 2 DAG, **j** 4 DAG, **k** 6 DAG, **l** 9 DAG. *Bars* = 2 mm (**a**, **b**, **e**, **f**), 0.5 mm (**c**, **d**, **i**), 0.1 mm (**g**), 0.2 mm (**h**), and 1 mm (**j**, **k**, **l**)

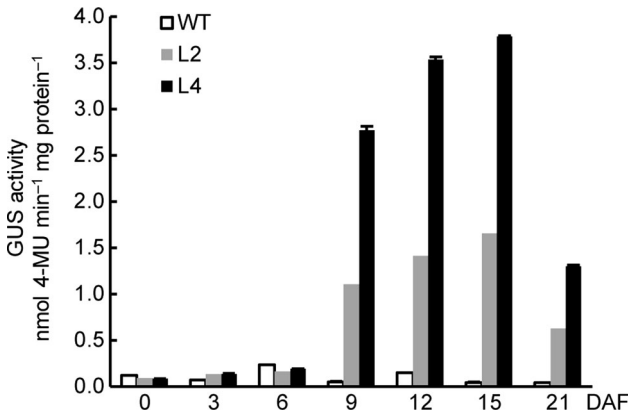

Fig. 5 A time course of GUS activity directed by the *JcMFT1* promoter during *Arabidopsis* seed development. Seeds from two homozygous transgenic lines (*L2* and *L4*) and wild-type (*WT*) were examined. The values are the average of four independent transgenic plants from each line. *Error bars* denote the SD from three replicates. *DAF* days after flowering

results demonstrate that the *JcMFT1* promoter characterized in this study is an ABA-inducible promoter.

Discussion

Although *MFT* was initially identified as a redundant floral inducer in *Arabidopsis* (Yoo et al. 2004), *MFT*-like genes have been proved to prefer being the regulator during seed development and germination in various plants (Chardon and Damerval 2005; Kikuchi et al. 2009; Nakamura et al. 2011). In this study, we identified two MFT genes in *Jatropha*, *JcMFT1* and *JcMFT2*. In agreement with previous studies, *JcMFT1* is highly expressed in seeds rather than other organs (Fig. 2a–c). We thus isolated *JcMFT1* promoter and evaluated it in *Arabidopsis*. Consisting with *JcMFT1* expression pattern in *Jatropha*, the *JcMFT1* promoter-GUS analysis in transgenic *Arabidopsis* showed the *JcMFT1* promoter was only activated in seeds of adult plants (Fig. 4a–f).

As shown in Fig. 2b, *JcMFT1* expression showed two peaks during seed development, one at the very early embryogenesis stage (7 DAP) and another at late seed-filling stage (42 DAP). Similar to *JcMFT1*, *TaMFT* expression profile in wheat also contained two peaks in seed development, one at the immature embryo stage and another after the physiological maturity stage (Nakamura et al. 2011). In *Picea abies*, *PaMFT1* and *PaMFT2* have similar expression patterns in embryos, in which both initial expressions were detected in early-stage embryos and the high levels remained during embryogenesis, and the expression level of *PaMFT2* declined evidently in mature embryos (Karlgren et al. 2011). In *Zea mays*, *MFT*-like *ZCN9* and *ZCN10* were expressed in embryos after 10 and 14 DAP, respectively (Danilevskaya et al. 2008). However, in *Citrus unshiu*, *CuMFT* was only expressed in mature seeds with undetectable expression in developing seeds (Nishikawa et al. 2008). In addition, like *MFT*-like genes in *Arabidopsis*, rice, and wheat (Danilevskaya et al. 2008; Nakamura et al. 2011; Xi et al. 2010), *JcMFT1* was also expressed in germinating seeds (Fig. 2c). In contrast to most of the *MFT*-like genes that are predominantly expressed in seeds, *DnMFT* from orchid was mainly

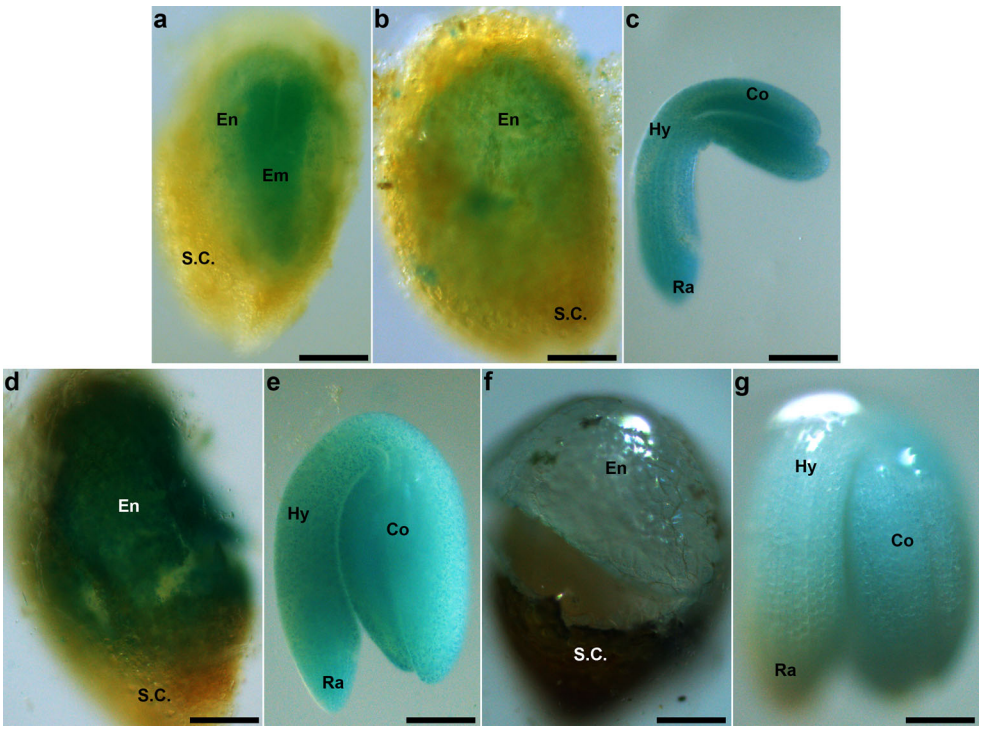

Fig. 6 GUS staining in dissected seeds from cotyledon stage to mature stage in transgenic *Arabidopsis* carrying a *JcMFT1 promoter-GUS* fusion. **a** Linear cotyledon stage. **b**, **c** Bent cotyledon stages. **d**, **e** Mature stages. **f**, **g** Post mature stages. *Em* embryo, *En* endosperm, *SC* seed coat, *Ra* radicle, *Hy* hypocotyl, *Co* cotyledon. *Bar* = 200 μm

expressed in auxiliary buds and leaves (Li et al. 2012). And tomato *MFT* homolog *SP2I* showed the constitutive expression in all organs (Carmel-Goren et al. 2003).

A variety of seed-specific elements were found in the *JcMFT1* promoter region (Fig. 3a), including an AACA core, a DOF core, a prolamin box, an RY repeat, and the SEF1, SEF3, and SEF4 motifs. The DOF core confers endosperm-specific expression in *Zea mays* (Yanagisawa 2000; Yanagisawa and Schmidt 1999). Although the AACA core and the prolamin box are involved in the regulation of endosperm-specific expression (Suzuki et al. 1998; Vicente-Carbajosa et al. 1997), they must function with GCN4 (Albani et al. 1997; Wu et al. 1998), which is an essential element in determining endosperm-specific expression (Wu et al. 2000). Considering the absence of GCN4 in the *JcMFT1* promoter, the DOF core, rather than the AACA core and the prolamin box, may confer promoter activity in the endosperm. SEFs (1 through 4) are embryo-specific factors involved in seed development (Lessard et al. 1991). In soybeans, SEF1 acts over the course of embryo development but most strongly at the mid to late stages; SEF3 and SEF4 also increase over the mid to late stages but decrease in desiccated seeds (Lessard et al. 1991). The SEF1, SEF3, and SEF4 binding sites are present in the *JcMFT1* promoter region (Fig. 3a). Because the *JcMFT1* promoter showed high activity at the mid to late stages of seed development of transgenic *Arabidopsis* (Fig. 5), we believe that the late embryogenesis elements, such as the SEF motifs and the RY repeat (Reidt et al. 2000) found in the *JcMFT1* promoter, may specify *JcMFT1* expression at mid to late stages of seed development.

In good agreement with our observation that *JcMFT1* expression and its promoter's activity are induced by ABA (Fig. 7), two ABA-responsive elements, a G-box and an RY repeat, were found in the *JcMFT1* promoter (Fig. 3a), which are recognized by *ABI3* (Ezcurra et al. 2000; Kim et al. 1997). It has been shown recently that during *Jatropha* seed development, *ABI3* expression starts at 29 DAP, peaks at 41 DAP and declines in mature seeds (Jiang et al. 2012). This expression pattern of *Jatropha ABI3* is very similar to that of *JcMFT1* in *Jatropha* seed development (Fig. 2b). Similarly, *PaVP1*, a homolog to *ABI3* in *Picea abies*, also showed a similar expression pattern during embryo development as observed for *PaMFT1* and *PaMFT2* (Karlgren et al. 2011). In *Arabidopsis*, *ABI3* is involved in the regulation of *MFT* expression in developing and germinating seeds (Xi et al. 2010). *ABI3* is expressed throughout *Arabidopsis* seed development, especially at mid to late stages; it is also transiently expressed beyond seed germination in young seedlings but exclusively in organs of embryonic origin (cotyledons and hypocotyls) (Parcy et al. 1994). Interestingly, the temporal and spacial

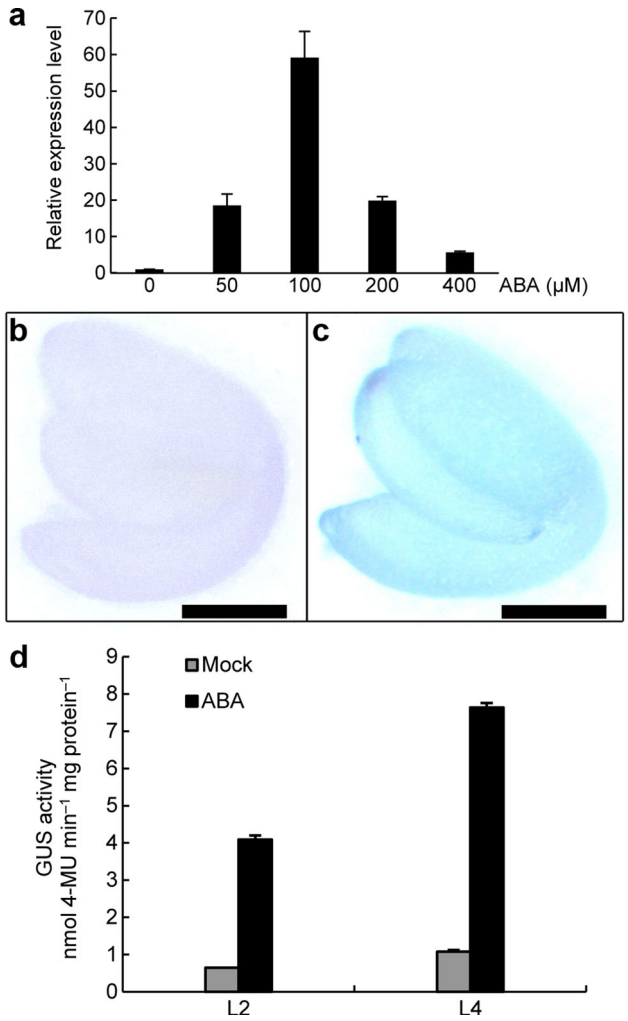

Fig. 7 *JcMFT1* expression and its promoter activity are induced by ABA. **a** *JcMFT1* expression levels in germinating *Jatropha* embryos treated with different concentrations of ABA. Equivalent qRT–PCR results were obtained from three biological replicates. The error bars denote the SD from triplicate technical replicates (n = 3). The values were normalized using the expression of the reference gene *JcGAPDH*. The relative mRNA level of the embryos treated with 0 μM ABA was set as the standard value of 1. **b**, **c** GUS assays in germinating seeds of *Arabidopsis* carrying a *JcMFT1 promoter-GUS* fusion. Histochemical GUS staining of embryos isolated from mock-treated (**b**) and 10 μM ABA-treated (**c**) germinating seeds. **d** Fluorometric GUS assay in germinating seeds of *Arabidopsis* carrying a *JcMFT1 promoter-GUS* fusion. Seeds from two homozygous transgenic lines (*L2* and *L4*) were examined. The values are the average of three independent transgenic plants from each line. *Error bars* denote the SD from three repeats. *Bars* = 0.1 mm (**b**, **c**)

activity of *JcMFT1* promoter in transgenic *Arabidopsis* (Figs. 4, 5) is also similar to the expression pattern of *Arabidopsis ABI3*. Taken together, these data suggest that *JcMFT1* promoter may mediate the regulation of *ABI3*, resulting in the presence of promoter activity in seeds and seedlings of transgenic *Arabidopsis*. As a seed-predominant promoter, the *JcMFT1* promoter has potential applications in plant genetic engineering and the functional analysis of genes in *Jatropha* and other plants.

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