

# Isolation and characterization of an ubiquitin extension protein gene (*JcUEP*) promoter from *Jatropha curcas*

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## Abstract

**Main conclusion** The *JcUEP* promoter is active constitutively in the bio-fuel plant *Jatropha curcas*, and is an alternative to the widely used *CaMV35S* promoter for driving constitutive overexpression of transgenes in *Jatropha*.

Well-characterized promoters are required for transgenic breeding of *Jatropha curcas*, a biofuel feedstock with great potential for production of bio-diesel and bio-jet fuel. In this study, an ubiquitin extension protein gene from *Jatropha*, designated *JcUEP*, was identified to be ubiquitously expressed. Thus, we isolated a 1.2 kb fragment of the 5' flanking region of *JcUEP* and evaluated its activity as a constitutive promoter in *Arabidopsis* and *Jatropha* using the  $\beta$ -glucuronidase (*GUS*) reporter gene. As expected, histochemical *GUS* assay showed that the *JcUEP* promoter was active in all *Arabidopsis* and *Jatropha* tissues tested. We also compared the activity of the *JcUEP* promoter with that of the cauliflower mosaic virus 35S (*CaMV35S*) promoter, a well-characterized constitutive promoter conferring strong transgene expression in dicot species, in various tissues of *Jatropha*. In a fluorometric *GUS* assay, the two promoters showed similar activities in stems, mature leaves and

female flowers; while the *CaMV35S* promoter was more effective than the *JcUEP* promoter in other tissues, especially young leaves and inflorescences. In addition, the *JcUEP* promoter retained its activity under stress conditions in low temperature, high salt, dehydration and exogenous ABA treatments. These results suggest that the plant-derived *JcUEP* promoter could be an alternative to the *CaMV35S* promoter for driving constitutive overexpression of transgenes in *Jatropha* and other plants.

**Keywords** Physic nut · Constitutive promoter · Ubiquitin · Transgenic · Stress · *CaMV35S*

## Introduction

Physic nut (*Jatropha curcas*), a small perennial tree, is an oil-producing plant whose seeds contain a high amount of oil. It has long been used around the world as a source of lamp oil and soap (Fairless 2007), and is now regarded as one of the best oilseed plants for renewable biodiesel production (Chakrabarti and Prasad 2012; Sato et al. 2011). To be an ideal bioenergy plant, the agronomic traits of *Jatropha*, such as seed yield and resistance to biotic and abiotic stresses, need to be improved (Chakrabarti and Prasad 2012; Divakara et al. 2010). However, the low genetic diversity of *Jatropha* (Cai et al. 2010; Tatikonda et al. 2009) means that it is difficult to make improvements by traditional breeding. Genetic engineering offers a feasible breeding strategy for *Jatropha* (de Argollo Marques et al. 2013; Sujatha et al. 2008), especially since several efficient transformation systems have been established (Joshi et al. 2010; Kumar et al. 2010, 2013; Li et al. 2008; Pan et al. 2010). The promoter is a crucial regulation factor determining whether a transgene can be expressed effectively to modify target

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plants. To date, though the *CaMV35S* (Odell et al. 1985) and *G10-90* (Ishige et al. 1999) promoters have been used in *Jatropha* transformation (Jha et al. 2013; Kim et al. 2014; Qin et al. 2014; Qu et al. 2012), the activities of these two promoters in *Jatropha* have not yet been evaluated in detail. Only *JcSDP1* promoter has been verified to be seed-specific active in *Jatropha* (Kim et al. 2014). Therefore, characterization of various promoters in *Jatropha*, including constitutive, tissue-specific or inducible promoters, is necessary for transgenic breeding.

In this paper, we isolated an ubiquitin promoter from *Jatropha*. Ubiquitin is a highly conserved protein of 76 amino acids that is present in all eukaryotes in two forms: polyubiquitin and ubiquitin extension protein. Polyubiquitin consists of several tandem ubiquitin monomers, and ubiquitin extension protein consists of a single ubiquitin monomer fused to a ribosomal protein (Burke et al. 1988; Christensen et al. 1992; Garbarino and Belknap 1994; Hoffman et al. 1991). Since ubiquitin has been proven to take part in many cellular processes (Finley et al. 1989; Glotzer et al. 1991; Perales et al. 2008), a number of ubiquitin promoters have been isolated from a variety of plants and applied as efficient constitutive promoters in plant genetic engineering.

In many monocots, especially cereal crops, ubiquitin promoters are notably more capable of driving transgene expression than the *CaMV35S* promoter. The GUS activity driven by the maize polyubiquitin *Ubi1* promoter is over tenfold higher than *CaMV35S* in maize, rice, barley and wheat (Christensen et al. 1992; Cornejo et al. 1993; Schledzewski and Mendel 1994). In rice, *Ubi1* is even more active than rice *Actin1*, but the rice polyubiquitin *RUBQ1*, *RUBQ2* and *rubi3* promoters cause two- to threefold higher activity than the *Ubi1* promoter (Sivamani and Qu 2006; Wang et al. 2000). The *RUBQ2* promoter also induced a higher level of GUS expression than the *Ubi1* promoter in sugarcane calli and leaves, while no GUS expression driven by the *CaMV35S* promoter was detected (Liu et al. 2003). Expression driven by the promoters of *ubi4* and *ubi9*, two sugarcane polyubiquitin genes, was high in sugarcane calli, and *ubi9* drove higher GUS activity than *Ubi1* in rice calli and regenerated plants (Wei et al. 2003). Polyubiquitin promoters isolated from floral monocots like *Lotus japonicus* and *Gladiolus* also direct higher GUS expression levels than the *CaMV35S* promoter (Joung and Kamo 2006; Kamo et al. 2012; Maekawa et al. 2008). However, the promoter of *uep1*, an ubiquitin extension protein isolated from oil palm, was less capable of driving GUS expression than *CaMV35S*, and the highest level was produced by the *Ubi1* promoter (Masura et al. 2010). When highly active ubiquitin promoters derived from monocots are used in dicots, some of them fail to direct transgene expression. The *Ubi1* promoter showed less than one-tenth of the capability of *CaMV35S* in

tobacco protoplasts (Christensen et al. 1992; Schledzewski and Mendel 1994). The switchgrass polyubiquitin promoters of *Pvubi1* and *Pvubi2*, which show strong activity not only in switchgrass calli but also in rice calli, roots, stems and leaves, failed to drive transgene expression in tobacco seedlings and mature plants, and only weak expression driven by *Pvubi2* was detected in reproductive organs (Mann et al. 2011). However, the tobacco polyubiquitin *Ubi.U4* promoter drove seven times higher GUS activity than *CaMV35S* in tobacco protoplasts and three times higher activity in 5-week-old plantlets, and was constitutively active in tobacco (Plesse et al. 2001). It is evident that dicot-derived ubiquitin promoters are the better choice for dicot applications and can be used instead of the widely used *CaMV35S* promoter. In embryogenic calli of olive and avocado, the sunflower polyubiquitin *ubB1* promoter is much better than the *CaMV35S* promoter (Chaparro-Pulido et al. 2014; Pérez-Barranco et al. 2009). The *Arabidopsis* ubiquitin extension protein *UBQ1* and *UBQ6* promoters drive GUS expression in all organs of tobacco with slightly lower expression in leaves than that driven by *CaMV35S* (Callis et al. 1990). The *ubi3* and *ubi7* promoters isolated from potato work in both the dicots potato and barrel medic (Confalonieri et al. 2010; Garbarino and Belknap 1994; Garbarino et al. 1995) and the monocot *Gladiolus* (Kamo et al. 2000).

Here, we report the isolation and characterization of the promoter of the *Jatropha* ubiquitin extension protein (*JcUEP*) gene. We found that the *JcUEP* promoter was constitutively active in both *Arabidopsis* and *Jatropha*, and maintained transgene expression in *Jatropha* under stress conditions. The endogenous *JcUEP* promoter is an attractive alternative to the *CaMV35S* promoter, a well-characterized and widely used constitutive promoter conferring strong transgene expression in dicot species, for driving constitutive overexpression of transgenes in *Jatropha*.

## Materials and methods

### Plant materials

*Jatropha curcas* plants cultivated in Xishuangbanna, Yunnan Province, China were used in this study 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.

### qRT-PCR analysis in *Jatropha*

A complete cDNA sequence (GenBank accession No. FM896034) of a ubiquitin extension protein (UEP) gene was identified from our embryo EST library of *Jatropha*

(Chen et al. 2011). Quantitative reverse transcriptase-polymerase chain reactions (qRT-PCR) were performed to examine the expression levels of *JcUEP* in various organs of *Jatropha* including 10-day-old seedlings, roots, stems, leaves, inflorescences, female flowers, male flowers, pericarps at 14 and 42 days after pollination (DAP), and seeds at 14 and 42 DAP. Total RNA was isolated (Ding et al. 2008) and reverse transcribed using the PrimeScript<sup>®</sup> RT reagent Kit with gDNA Eraser (TAKARA). qRT-PCR was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (TAKARA) on the Roche 480 Real-time PCR detection system (Roche Diagnostics). All gene expression data obtained by qRT-PCR were normalized to the combined expression of *JcActin* and *JcGAPDH* (Zhang et al. 2013). The primers used in qRT-PCR are listed in Table 1.

#### Cloning of the 5' flanking region of *JcUEP*

The 5' flanking region upstream of the translation start codon of *JcUEP* was isolated from *Jatropha* genomic DNA

by genome walking (Siebert et al. 1995). For nested PCR, the *JcUEP* gene-specific primers GSP1, GSP2, GSP3, GSP4 and adaptor primers AP1, AP2 were used. The *JcUEP* promoter was amplified by PCR using the primers XT381 (forward) and XT395 (reverse) carrying *Hind*III and *Bam*HI restriction sites, respectively, and was cloned into the pGEM-T Easy vector for sequencing. The putative *cis*-acting elements of the *JcUEP* promoter were analyzed with the PLACE database (Higo et al. 1999). The primers used in genome walking are listed in Table 1.

#### Construction of the promoter-GUS fusion

To generate the *JcUEP:GUS* plasmid, *Hind*III and *Bam*HI were used to digest pBI101 (Jefferson et al. 1987) and the pGEM-T Easy vector containing the 1.2 kb *JcUEP* promoter, respectively. The two fragments were linked using T4 ligase (Promega). The resulting construct, *JcUEP:GUS* (Fig. 2b) was transferred into *A. tumefaciens* EHA105 and LBA4404 by electroporation (GenePulser Xcell, Bio-Rad).

**Table 1** Sequences of the primers used in this study

Name	Sequence (from 5' to 3')	Feature
AP1	GTAATACGACTCACTATAGGGC	Adaptor primer for genome walking
AP2	ACTATAGGGCACGCGTGGT	Adaptor primer for genome walking
GSP1	TGTCCTGGATCTTGGCCTTGACATTAT	<i>JcUEP</i> gene-specific primer for genome walking
GSP2	CAGAGGAGAGGAGCAGTAGCCGAAG	<i>JcUEP</i> gene-specific primer for genome walking
GSP3	CGTTTCGGAGTTATACGGAGATTGAAC	<i>JcUEP</i> gene-specific primer for genome walking
GSP4	TATCCATTTGTCGCCCGCTATCTTCTT	<i>JcUEP</i> gene-specific primer for genome walking
XK191	CTCCTCTCAACCCCAAAGCCAA	<i>JcActin</i> gene primer for qRT-PCR
XK192	CACCAGAATCCAGCACGATACCA	<i>JcActin</i> gene primer for qRT-PCR
XT95	GCTGCTAAGGCTGTTGGGAA	<i>JcGAPDH</i> gene primer for qRT-PCR
XT96	GACATAGCCCAATATTCCTTCAG	<i>JcGAPDH</i> gene primer for qRT-PCR
XK414	ATCCCTGCTTCGGCTACTGCTCC	<i>JcUEP</i> gene primer for qRT-PCR
XK415	GTCTGGAGGGATGCCTTCCTTGTC	<i>JcUEP</i> gene primer for qRT-PCR
XT381	CCCAAGCTTATCTAACATATTATGCGTC	For cloning the full-length promoter and construction of <i>JcUEP:GUS</i> , added <i>Hind</i> III site was underlined
XT395	CGCGGATCCGAGAGGAGCAGTAGCCGAA	For cloning the full-length promoter and construction of <i>JcUEP:GUS</i> , added <i>Bam</i> HI site was underlined
XK310	TACAGGTGACCAGCTCGAATTTCC	For TAIL-PCR amplifying the right flanking sequence
XK311	ATTGAATCCTGTTGCCGGTCTTG	For TAIL-PCR amplifying the right flanking sequence
XK312	GAGTCCCGCAATTATACATTTAATACGC	For TAIL-PCR amplifying the right flanking sequence
XK314	GGATACCGAGGGGAATTTATGGAA	For TAIL-PCR amplifying the right flanking sequence
XK315	TGACCTTAGGCGACTTTTGAACG	For TAIL-PCR amplifying the right flanking sequence
XK316	CAGTTCCAAACGTAACCGGCTTG	For TAIL-PCR amplifying the right flanking sequence
AD1	ASCWGNTSAGNTSAGG	For TAIL-PCR amplifying the right flanking sequence
AD2	TGNCASTCWGNANTCG	For TAIL-PCR amplifying the right flanking sequence
AD3	GWANCTNASTCGNGTT	For TAIL-PCR amplifying the right flanking sequence
AD4	TGNWCWGNTSANSACT	For TAIL-PCR amplifying the right flanking sequence
XT450	ACTCATTACGGCAAAGTGTGGGTCA	For amplifying the GUS probe of Southern hybridization
XT451	GAGTTCATAGATAACCTTACCCG	For amplifying the GUS probe of Southern hybridization

The strain EHA105 harboring the construct was used to transform *Arabidopsis*; the strain LBA4404 harboring the construct, pCAMBIA2301 (containing the *CaMV35S* promoter, positive control) or pBI101 (promoter-less, negative control, NC) was used to transform *Jatropha*.

#### Plant transformation

*Jatropha* transformation was performed following Pan et al. (2010) with several modifications. After sterilization with 75 % (v/v) ethanol for 30 s, the mature seeds of *Jatropha* were then sterilized with 10 % (v/v) sodium hypochlorite for 20 min. The embryos were removed from the sterilized seeds, and the cotyledons were cut off at the base leaving 3/4 papery cotyledons as explants for co-cultivation with *Agrobacterium*. In co-cultivation, 50  $\mu$ M acetosyringone was added to the MS-Jc1 medium (Pan et al. 2010). After 2-day co-cultivation, the explants were cultured on MS-Jc1 medium with 100 mg/L timentin for a 10-day recovery, and then were subcultured on shoot-inducing medium (SIM, MS-Jc1 medium with 40 mg/L kanamycin and 100 mg/L timentin). After selection, the regenerated shoots were transferred onto rooting medium (RM, half strength (1/2) MS medium with 0.2 mg/L IBA, 0.1 mg/L NAA and 100 mg/L timentin). Finally, the putative *Jatropha* transformants were examined by TAIL-PCR and histochemical GUS assay, and the positive transgenic plants were cultivated in soil. *Arabidopsis* transformation was performed by the floral dip method (Clough and Bent 1998).

#### TAIL-PCR analysis

Thermal asymmetric interlaced PCR (TAIL-PCR) was performed with genomic DNA isolated from leaves of the putative *Jatropha* transformants. The procedure was performed as described by Liu et al. (1995). Three specific primers each were designed for pCAMBIA2301 and pBI101 based on the right border sequences of their T-DNAs. Together with arbitrary degenerate (AD) primers (AD1, AD2, AD3 and AD4), the T-DNA-specific primers XK310, XK311 and XK312 were used to examine the *CaMV35S*:*GUS* transformants, and XK314, XK315 and XK316 were used to examine the *JcUEP*:*GUS* transformants. The primers used here are listed in Table 1. After the tertiary PCR reaction, the TAIL-PCR products were sequenced, and the resulting sequences were used to perform BLASTN searches against the *Jatropha* Genome Database (<http://www.kazusa.or.jp/jatropha/>).

#### Southern blot analysis

The total genomic DNA (8  $\mu$ g) isolated from the leaves of transgenic plants was digested with restriction enzyme

*EcoRI* and *XhoI*, whose cut sites are not inside the probe *GUS* fragment and separated on 0.8 % agarose gel. Then the gel was processed and transferred to a Hybond-N<sup>+</sup> membrane (Roche, Mannheim, Germany) following the standard procedure (Sambrook et al. 2001). The probe of a 483-bp *GUS* fragment was prepared with PCR DIG Probe Synthesis Kit (Roche) using specific primer pairs of XT450 and XT451 (Table 1). Hybridization was performed with DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the instruction.

#### Stress treatment

Ten-day-old seedlings of the T1 generation of transgenic *Jatropha* harboring *JcUEP*:*GUS* were subjected to stress treatments. One of the cotyledons was used for the GUS assay before the treatment, and another cotyledon of the same seedling for the assay after the treatment. From three different T0 plants, three seedlings from each T0 plant, were used for each stress treatment. Total nine seedlings (T1 plants) from each three independent transgenic lines, three seedlings from each line, were used for 4 °C (lines A1, A5, and A9), NaCl (lines A1, A6, and A9), PEG (lines A1, A5, and A6), and ABA (lines A1, A3, and A6) treatment, respectively. Seedlings were incubated in half-strength MS liquid medium with 200 mM NaCl, 30 % PEG6000 and 100  $\mu$ M ABA for high salt, dehydration and exogenous ABA treatments, respectively; seedlings were kept at 4 °C for low temperature treatment. All treatments lasted 2 days until the seedlings started drooping.

#### Histochemical and fluorometric GUS assay

For histochemical GUS staining, various tissues of transgenic *Jatropha* and *Arabidopsis* were incubated in GUS assay buffer with 50 mM sodium phosphate (pH 7.0), 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, 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 stereo-microscopy (Leica M80).

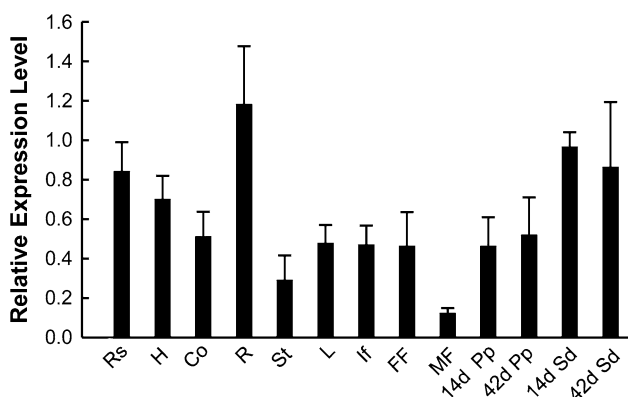
To examine the activity of the *JcUEP* and *CaMV35S* promoters in transgenic *Jatropha*, fluorometric GUS assay was performed following the protocol described by Jefferson et al. (1987), modified by adding 2 mM MUG in the reaction buffer. The fluorescence was examined with a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation). The protein concentrations of plant extracts were measured following Bradford (1976). T0 plants from each six independent transgenic lines of *JcUEP*:*GUS* (lines A1, A3, A5, A6, A9, and A10) and *CaMV35S*:*GUS* (lines D1, D3, D5, D7, D12 and D15) were used for this experiment.

## Result

### Expression pattern of *JcUEP* in *Jatropha*

Since ubiquitin is a highly conserved protein found in all eukaryotes, we identified an ubiquitin extension protein cDNA (GenBank accession No. FM896034) from our *Jatropha* embryo EST library (Chen et al. 2011) and designated it *JcUEP*. *JcUEP* encoded a single ubiquitin monomer (76 amino acids) fused to a ribosomal protein (80 amino acids). Compared with ubiquitin extension proteins in other plant species, this 156 amino acid sequence showed 100 % identity to *Sesamum indicum* SiUBQ5 (GenBank accession No. AFJ42574), and 99 % identity to *Hevea brasiliensis* HbUEP1 (GenBank accession No. ABN72579), *Gossypium hirsutum* GhUBQ7 (GenBank accession No. AAZ83341) and *Nicotiana tabacum* NtUBQ2 (GenBank accession No. AAZ53360) (data not shown).

To investigate whether *JcUEP* was expressed constitutively in *Jatropha*, qRT-PCR analysis was performed with total RNA extracted from various tissues. The results showed that *JcUEP* was expressed in all tested tissues (Fig. 1). The highest expression level was detected in roots; expression was slightly lower in seeds at 14 and 42 days after pollination (DAP). *JcUEP* was also highly expressed in 10-day-old seedlings, leaves, inflorescences, female flowers and pericarps at 14 and 42 DAP. Low expression levels were detected in stems and male flowers. Although the expression levels varied among different



**Fig. 1** Expression pattern of *JcUEP* in *Jatropha*. Samples from 10-day-old seedlings: roots (RS), hypocotyls (H), and cotyledons (Co); samples from adult plants: roots (R), stems (St), leaves (L), inflorescences (If), female flowers (FF), male flowers (MF), pericarps at 14 days after pollination (DAP) (Pp 14 days) and 42 DAP (Pp 42 days), and seeds at 14 DAP (Sd 14 days) and 42 DAP (Sd 42 days). qRT-PCR results were obtained from four biological replicates. The error bars denote the SD. The values were normalized to the combined expression of *JcActin* and *JcGAPDH*

organs, *JcUEP* was expressed throughout the life cycle of *Jatropha*.

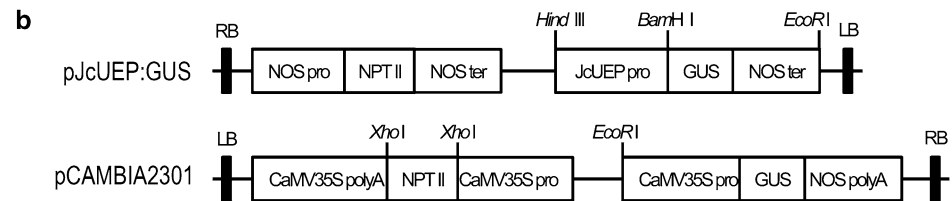
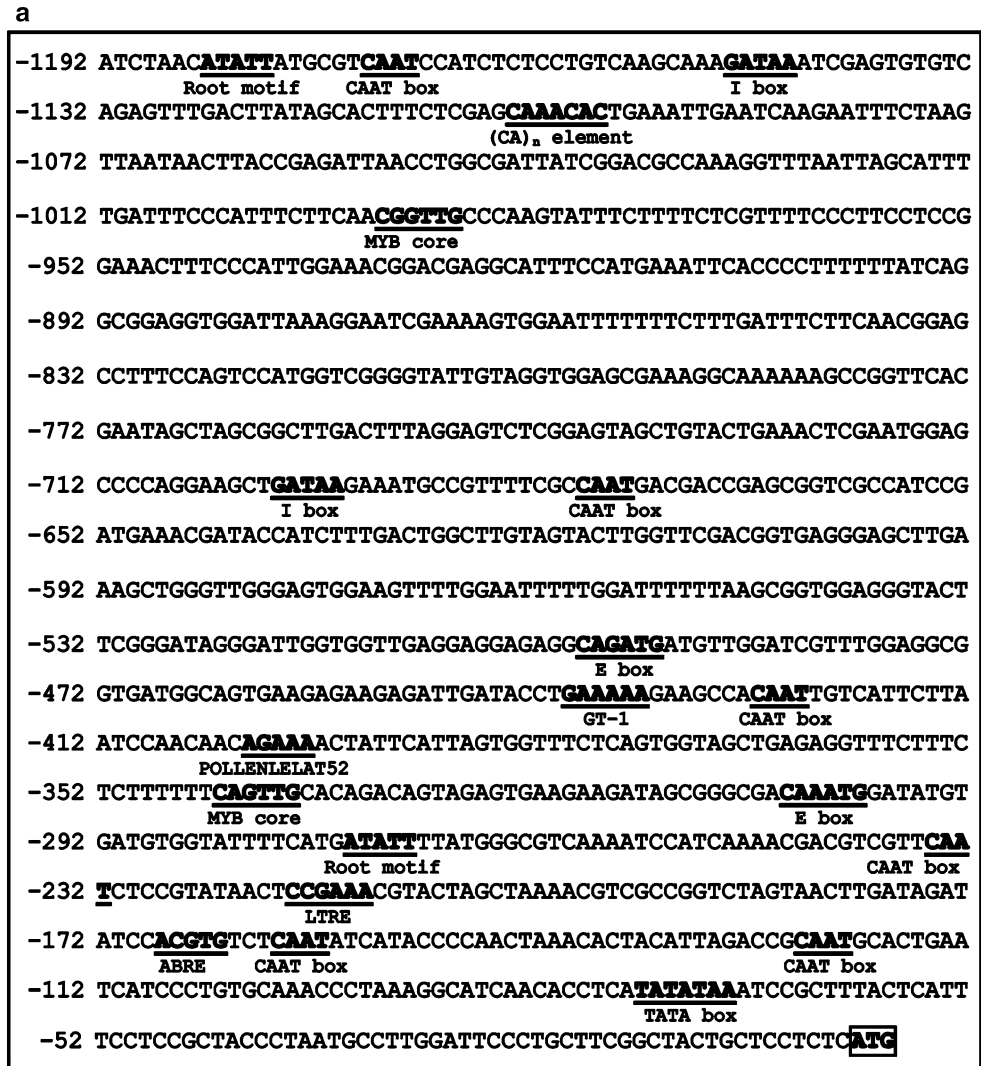
### Isolation and sequence analysis of the *JcUEP* promoter

Based on the expression data, the 1.2-kb *JcUEP* promoter fragment (GenBank accession no. KJ202645) was isolated from *Jatropha* by genome walking and was analyzed for putative *cis*-acting elements using the PLACE database (Higo et al. 1999). The promoter sequence and putative plant regulatory elements are shown in Fig. 2a. The analysis revealed that various putative plant regulatory elements are present in the *JcUEP* promoter region. There were some tissue-specific elements, such as the root motif (ATATT) for root expression (Elmayan and Tepfer 1995), the I box (GATAA) for leaf expression (Donald and Cashmore 1990), POLLEN1LELAT52 (AGAAA) for pollen expression (Twell et al. 1991), and the (CA)<sub>n</sub> element (CNAACAC) and E box (CANNTG) for seed expression (Ellerström et al. 1996; Kawagoe and Murai 1992; Stålborg et al. 1996). In addition, the *JcUEP* promoter contained other regulatory elements involved in responses to environmental changes and hormone signaling. The I box, besides its involvement in leaf-specific expression, is also an important light-responsive element (Manzara et al. 1991; Rose et al. 1999). LTRE (CCGAAA) is known to be a low temperature-responsive element (Dunn et al. 1998). GT-1 (GAAAAA) and MYBCORE (CNGTTR) are involved in salt- and drought-induced regulation (Park et al. 2004; Urao et al. 1993). The ABRE (ACGTG), a well-known hormonal element, is involved in the response to abscisic acid (ABA) (Ezcurra et al. 1999; Sibénil et al. 2001). These results were taken as an indication that multiple *cis*-acting elements conferred constitutive activity on the *JcUEP* promoter.

### Characterization of *JcUEP* promoter activity in transgenic *Arabidopsis*

To test the promoter activity, *JcUEP*:*GUS* (Fig. 2b) was transformed into *Arabidopsis* for preliminary analysis. *GUS* staining was examined in the T0 generation of six transgenic lines. As shown in Fig. 3, the 1-week-old seedlings exhibited strong *GUS* staining in emerging true leaves, cotyledons, and roots, especially in the tips of roots, and faint staining was observed in hypocotyls. *GUS* staining was also observed in different tissues of adult plants, including roots, leaves, inflorescence stems, flowers and siliques. The staining results verified that the *JcUEP* promoter was able to drive transgene expression and was constitutively active in transgenic *Arabidopsis*.

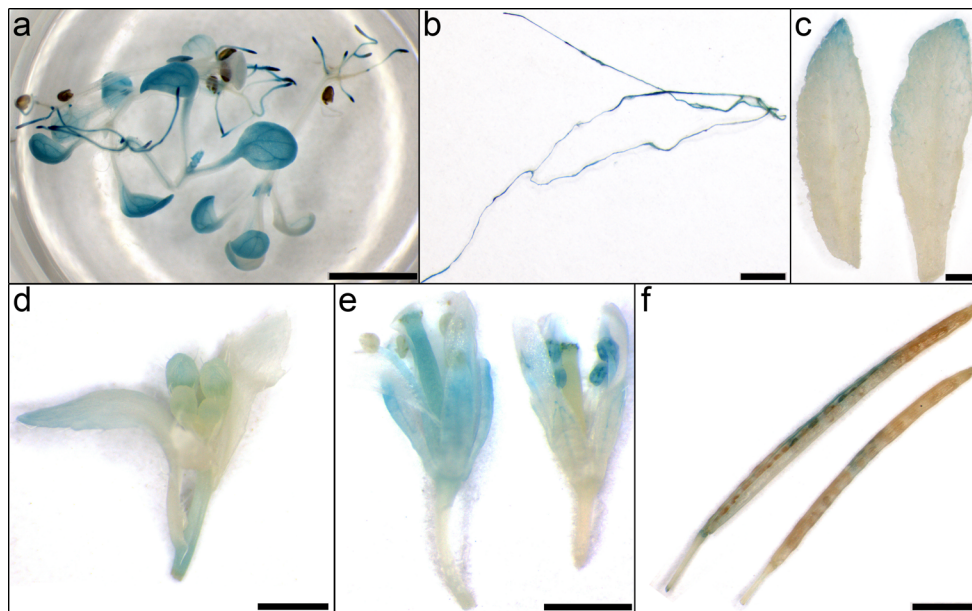
**Fig. 2** *JcUEP* promoter-reporter gene construct. **a** The nucleotide sequence of the *JcUEP* promoter. The A of the start codon ATG (*bold and boxed*) of *JcUEP* is numbered as +1. Putative regulatory elements on both strands are shown in *bold and underlined*. **b** A schematic of the T-DNA regions of the *JcUEP:GUS* and pCAMBIA2301 (GenBank accession No. AF234316) binary vectors used for transformation



Generation and molecular analysis of transgenic *Jatropha*

According to the staining results mentioned above, *JcUEP:GUS* was transformed into *Jatropha* to determine whether *JcUEP* promoter worked as in *Arabidopsis*. Transformed plantlets were generated by kanamycin selection. Regenerated leaves from eight, ten and six independent transgenic lines transformed with *JcUEP:GUS*, *CaMV35S:GUS* and the negative control pBI101, respectively, were used for histochemical GUS assay. As expected, GUS-positive transformants were stained blue

while the negatives were not (data not shown). To confirm stable transgene integration, the negative control and GUS-positive transformants were examined by TAIL-PCR. The T-DNA right border insert was used for integration analysis. The T-DNA right borders from pBI101 and pCAMBIA2301, and two insert events selected randomly from three transformation events are displayed in Table 2. The sequences of the junctions obtained from the negative control and *JcUEP:GUS* transformants are listed below the T-DNA right border of pBI101, and those from *CaMV35S:GUS* are listed below pCAMBIA2301. The T-DNA integration sites were determined as shown in the



**Fig. 3** Histochemical GUS staining of transgenic *Arabidopsis* harboring the *JcUEP:GUS* fusion. **a** One-week-old seedlings; various organs of adult plants: **b** roots, **c** leaves, **d** inflorescence stems, **e** flowers, **f** siliques bearing seeds. Bars: **a, c, f** = 2 mm; **b, d, e** = 1 mm

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

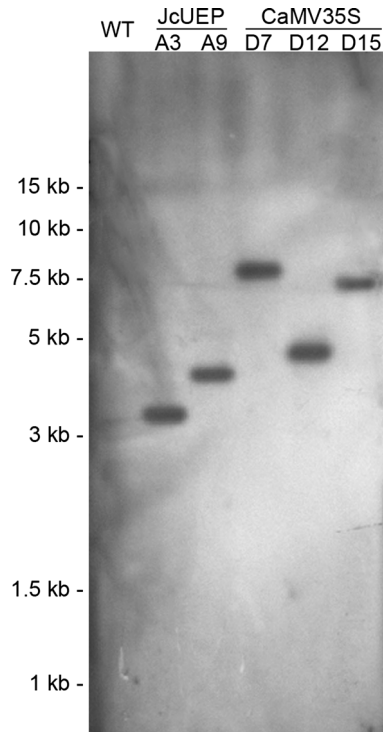
Plasmid	No. <sup>a</sup>	Right boundary T-DNA/plant genomic DNA junctions
pBI101		--CAGTTTAAACTATCAGTGTTT <i>TGACAGGATATATTGGCGGGTAAAC</i> --
	F1	--CAGTTTAAACTATCAGTGTTT <i>TGATC</i> <b>TTTCTAGCCCTTACTCTAGGAAAGAACCAG</b> --
	F5	--CAGTTTAAACTATCAGTGTTT <i>TGAAATATTAATATCTTATTACCAAATGTC</i> <b>CCCATGTC</b> --
	A6	--CAGTTTAAACTATCAGTGTTT <i>TGAGACTTCTATGCCCCTCTCCTTAACTTGAGGTCAA</i> --
	A9	--CAGTTTAAACTATCAGTGTTT <i>TGATGACAGAATGACACGAGATTATGAAATTGAAAT</i> --
pCAMBIA2301		--AATTAAACTATCAGTGTTT <i>TGACAGGATATATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTTA</i> --
	D7	--AATTAAACTATCAGTGTTT <b>TTGATTTGTCAAAGCATAACAACTTTCTCCATTT</b> --
	D15	--AATTAAACTATCAGTGTTT <b>TGACTTGGTTCGATTTAGAACTGATTGGCTCAATTC</b> --

<sup>a</sup> Serial number of transgenic *Jatropha* plants with construct pBI101 (F1 and F5, negative control), *JcUEP:GUS* (A6 and A9), or *CaMV35S:GUS* (D7 and D15)

T-DNA/*Jatropha* genomic DNA junction regions. The loss of the right border sequence observed here has also occurred in TAIL-PCR detection in transgenic rice, *Arabidopsis*, leek and even fungi (Eady et al. 2005; Hiei et al. 1994; Lee and Bostock 2006; Liu et al. 1995). It seems that the right border is easily deleted in these species.

In addition, we performed Southern blot to further characterize the transformants. Since *EcoRI* and *XhoI* restriction sites are not inside the probe *GUS* fragment (Fig. 2b), the number of bands hybridizing to digoxigenin (DIG)-labeled *GUS* cDNA on Southern blot analysis of *EcoRI* and *XhoI*-digested DNA should give a good estimate of the transgene copy number in transgenic plant genome.

The result (Fig. 4) showed that the *GUS* fragment had been integrated into the genome of *JcUEP:GUS* and *CaMV35S:GUS* transformants, respectively. Different sizes of hybridizing bands shown in Fig. 4 implied that the various transgenic lines resulted from independent transformation events. Single-copy insertions were observed in the transgenic lines analyzed (Fig. 4), indicating single-copy transgene insertions in these transformants. Taken together, these results indicated that the transgenes were definitely integrated into the genome of *Jatropha* transformants, and the integration sites in the *Jatropha* genome were different between insert events, indicating that they were independent lines for each transformation event. The transgenic plantlets were grown in soil for further analysis.



**Fig. 4** Southern blot analysis of transgenic *Jatropha* plants. Eight  $\mu$ g of total genomic DNA was digested with restriction enzymes *Eco*RI and *Xho*I, and fractionated on a 0.8 % agarose gel. The DNA was blotted onto Hybond-N<sup>+</sup> membrane and hybridized to a digoxigenin (DIG)-labeled *GUS* probe. Lane 1, wild type *Jatropha*; lanes 2 and 3, transgenic lines (A3 and A9) with *JcUEP:GUS*; lanes 4–6, transgenic lines (D7, D12 and D15) with *CaMV35S:GUS*

#### Characterization of *JcUEP* promoter activity in transgenic *Jatropha*

To evaluate the constitutive capability of the *JcUEP* promoter, histochemical staining was first performed on various tissues of transgenic *Jatropha* including roots, stems, young leaves, mature leaves, inflorescences, female flowers, male flowers, fruits at 12 DAP and seeds at 25 DAP from the T0 generation and 3-day-old seedlings from the T1 generation. pCAMBIA2301 (*CaMV35S* promoter) was used as a positive control, as the *CaMV35S* promoter was capable of driving transgene expression in *Jatropha* (Kumar et al. 2010; Pan et al. 2010). pBI101 (promoter-less) was used as a negative control. All of the examined tissues from transgenic plants with *JcUEP:GUS* and *CaMV35S:GUS* were stained while the negative controls were not (Figs. 5, 6). This indicated that the *JcUEP* promoter was also constitutively active in *Jatropha* as in *Arabidopsis*.

Next, the activity of the *JcUEP* promoter was further evaluated in *Jatropha* by fluorometric GUS assay and compared with the *CaMV35S* promoter simultaneously. Different tissues from six independent lines of the T0 generation were used for GUS detection and the results are

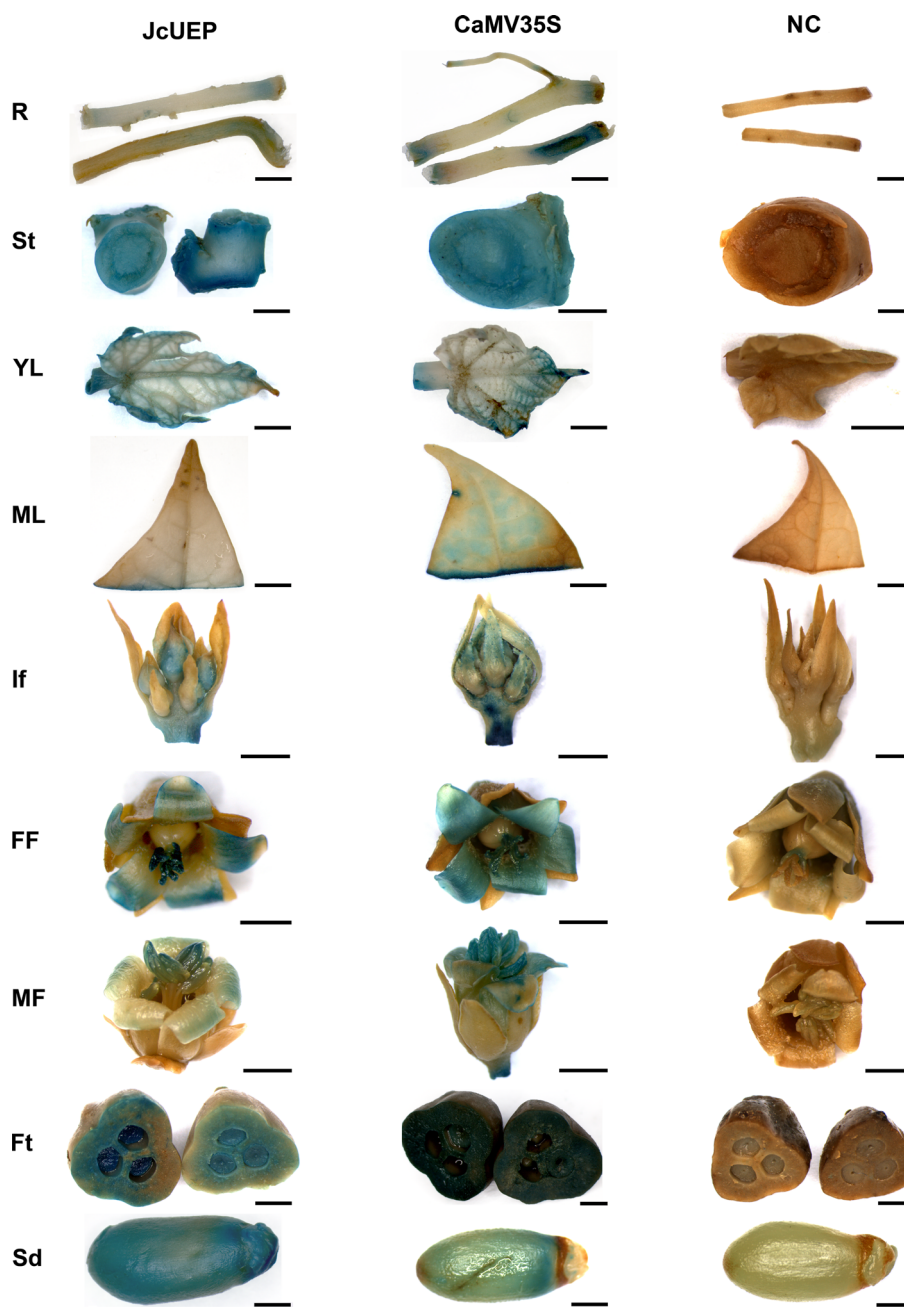
shown in Table 3. We found that the *GUS* expression levels driven by the *CaMV35S* promoter were higher than those driven by the *JcUEP* promoter in most tissues. The *GUS* expression levels in the roots, young leaves, inflorescences, male flowers, fruits (12 DAP) and pericarps (25 DAP) of transgenic plants with *CaMV35S:GUS* were at least threefold higher than in transgenic plants with *JcUEP:GUS*, especially in young leaves and inflorescences, which were 12.9- and 15.2-fold higher, respectively. In seeds (25 DAP), the *CaMV35S* promoter induced only 1.7-fold higher expression than the *JcUEP* promoter. However, the expression levels of both were almost equal in stems, mature leaves and female flowers. Although the *CaMV35S* promoter was more effective than the *JcUEP* promoter in some tissues, we noticed that its activity varied markedly among different tissues. In transgenic plants with *CaMV35S:GUS*, the highest *GUS* expression level was detected in fruits (12 DAP), followed by inflorescences and roots. The levels in young leaves, pericarps and seeds (25 DAP) were similar and slightly lower than in roots. In stems, female flowers and male flowers, the *GUS* expression levels were low, and the lowest activity was detected in mature leaves. There was great variation (27.6-fold) between the highest and the lowest activities. Compared with the *CaMV35S* promoter, the *GUS* expression driven by the *JcUEP* promoter varied less among these tissues. The highest level was also detected in fruits (12 DAP), followed by seeds (25 DAP). The expression levels in other tissues were very similar, and young leaves showed the lowest level. The variation of 13.8-fold between the highest and the lowest expression levels was half of that produced by the *CaMV35S* promoter.

#### Activity of the *JcUEP* promoter in transgenic *Jatropha* under stress conditions

Since the *JcUEP* promoter contained some stress-responsive elements, further work was carried out to investigate whether it was active under various stress conditions. *GUS* expression was examined by fluorometric assay in 10-day-old seedlings of the T1 generation that were treated with low temperature (4 °C), high salt, dehydration and exogenous ABA. As shown in Fig. 7, *GUS* expression levels did not change obviously in these stress treatments. In low temperature, high salt and dehydration conditions, the levels increased slightly. These results indicated that the *JcUEP* promoter was capable of maintaining transgene expression in a severe stress environment. This also further confirmed that the *JcUEP* promoter was constitutively active in *Jatropha*. It is worthy to note that significant variations were found in GUS activity in control among the different stress treatments (Fig. 7), which are likely due to the use of different transgenic lines.



**Fig. 5** Histochemical GUS staining in various tissues of transgenic *Jatropha* (T0) harboring *JcUEP:GUS*, *CaMV35S:GUS* and pBI101 (NC). *R* roots, *St* stems, *YL* young leaves, *ML* mature leaves, *If* inflorescences, *FF* female flowers, *MF* male flowers, *Ft* fruits at 12 days after pollination (DAP), *Sd* seeds at 25 DAP. All bars 2 mm

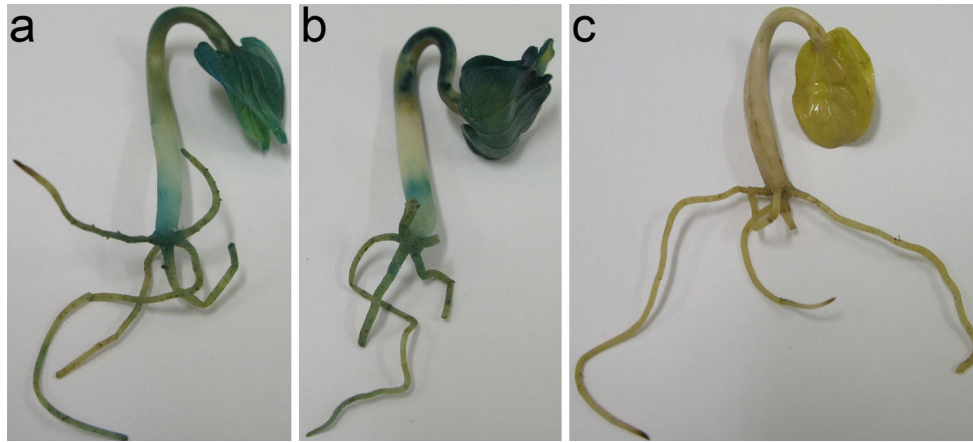


**Discussion**

As the promoter initiates gene transcription and regulates gene expression temporally and spatially, it plays an important role in plant genetic engineering. A good knowledge of the pattern of promoter activity is necessary for gene function studies and transgenic breeding (Hernandez-Garcia et al. 2009; Rooke et al. 2000). At present, research on *Jatropha* genetic engineering is just a beginning, so the available information for promoter analysis in *Jatropha* is very limited. In this work, to find new effective promoters, we isolated the *JcUEP* promoter from *Jatropha*

and characterized it in *Arabidopsis* and *Jatropha*. In addition, to our knowledge, this is the first time the *CaMV35S* promoter has been characterized in various tissues of *Jatropha*.

We found that *JcUEP* has a constitutive expression pattern in *Jatropha* with varying levels in different tissues (Fig. 1). Similar to our findings, ubiquitin extension protein genes from other plant species also exhibit varied expression patterns. In tomato, *ubi3* RNA was preferentially accumulated in green and immature tissues over yellow and mature tissues (Hoffman et al. 1991). As with tomato *ubi3*, the oil palm *uep1* transcript was more abundant in young



**Fig. 6** Histochemical GUS staining in 3-day-old seedlings of transgenic *Jatropha* (T1) harboring *JcUEP:GUS* (a), *CaMV35S:GUS* (b) and pBI101 (NC) (c)

**Table 3** GUS activities driven by the *JcUEP* and *CaMV35S* promoters in various tissues of transgenic *Jatropha* (T0)

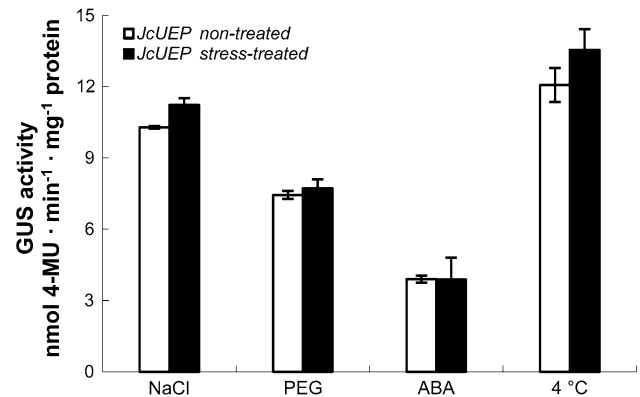
	<i>JcUEP</i>	<i>CaMV35S</i>	NC <sup>a</sup>
Root	13.62 ± 7.91	103.25 ± 219.86	0.26 ± 0.18
Stem	22.34 ± 24.46	24.70 ± 54.58	0.45 ± 0.36
Young leaf	4.96 ± 4.03	64.31 ± 88.05	0.06 ± 0.03
Mature leaf	7.07 ± 10.46	6.94 ± 8.28	0.10 ± 0.08
Inflorescence	7.62 ± 4.26	116.28 ± 165.32	0.18 ± 0.19
Female flower	13.14 ± 21.24	18.08 ± 26.41	0.24 ± 0.23
Male flower	7.95 ± 6.83	24.02 ± 36.41	0.38 ± 0.14
Fruit (12 DAP)	68.94 ± 105.10	191.75 ± 202.24	1.53 ± 1.10
Pericarp (25 DAP)	14.94 ± 15.38	80.55 ± 55.46	0.52 ± 0.47
Seed (25 DAP)	36.15 ± 19.72	62.63 ± 45.94	0.06 ± 0.04

The values (nmol 4-MU min<sup>-1</sup> mg<sup>-1</sup> protein) are averages of six independent transgenic lines (±SD). Three biological replicates were prepared for each transgenic line

DAP days after pollination

<sup>a</sup> NC is the negative control harboring pBI101

tissues. High levels of *uep1* expression were detected in the early stages of mesocarp development and the levels were slightly decreased at later stages; the expression levels were also high in roots from plantlets, stems, young leaves, flowers and embryoids, but low in green mature leaves (Masura et al. 2010). However, *JcUEP* did not show a preference for young tissues. The expression level of *JcUEP* in mature roots was higher than that in young roots from 10-day-old seedlings. The level in pericarps at early developmental stage was slightly lower than in later stage, while the levels in the seeds showed an opposite pattern. In potato, though *ubi3* expression was highest in the shoot tip, the level in old leaves was in excess of that in mature leaves (Garbarino and Belknap 1994).



**Fig. 7** Effects of stress treatments on GUS activity directed by the *JcUEP* promoter in 10-day-old seedlings of transgenic *Jatropha* (T1). The values are averages of three independent transgenic lines. Error bars denote the SE from three replicates. ‘*JcUEP* non-treated’ and ‘*JcUEP* stress-treated’ denote the GUS activity in the seedlings harboring *JcUEP:GUS* before and after stress treatment

Although the expression levels varied among different tissues, *JcUEP* was ubiquitously expressed in *Jatropha*. Therefore, the 1.2 kb *JcUEP* promoter was isolated as a potential constitutive promoter. After characterization in transgenic *Arabidopsis* and *Jatropha*, the *JcUEP* promoter showed the expected capability to drive transgene expression in all tissues tested. *GUS* expression driven by the *CaMV35S* promoter was also observed throughout the transgenic *Jatropha*. Sequence analysis of the *CaMV35S* promoter has shown that the cooperation of multiple *cis*-acting elements is required to confer the great activity of the promoter (Fang et al. 1989). Perhaps, the cooperation of multiple tissue-specific elements existing in the promoter region causes the *JcUEP* promoter to be active in various tissues. Furthermore, the *JcUEP* promoter retained its activity in low temperature, high salt, dehydration and

exogenous ABA conditions (Fig. 7). Among the two types of ubiquitin promoters, polyubiquitin and ubiquitin extension protein promoters, most polyubiquitin promoters had been shown to be induced under stress. For example, maize polyubiquitin promoter *Ubi-1* was induced by heat shock (Takimoto et al. 1994), potato polyubiquitin promoter *ubi7* activated by wounding (Garbarino et al. 1995), and sugarcane polyubiquitin promoter *ubi4* induced by heat shock (Wei et al. 2003). Most ubiquitin extension protein promoters, however, were not significantly induced under stress, e.g., potato ubiquitin extension protein promoter *ubi3* was not activated by wounding in the tuber (Garbarino and Belknap 1994), and oil palm ubiquitin extension protein promoter *uep1* was not significantly induced by exogenous auxin and ABA treatments (Masura et al. 2010). Taken together, we believe that the combined regulation of multiple elements ensures the constitutive activity of the *JcUEP* promoter.

Though the *JcUEP* and *CaMV35S* promoters drove *GUS* expression in all tissues of *Jatropha*, the expression patterns differed except in fruit (12 DAP), which showed the highest activities under both (Table 3). In transgenic *Jatropha*, the *CaMV35S* promoter seems to be preferentially active in young tissues. *GUS* activity in young leaves was much higher than in mature leaves, and the activity in inflorescences was significantly greater than in flowers. The *CaMV35S* promoter also showed the same preference in rice, soybean and oil palm (Battraw and Hall 1990; Hernandez-Garcia et al. 2009; Masura et al. 2010). The ‘preference’ of the *CaMV35S* promoter may have caused the large variation of *GUS* activity among different tissues in *Jatropha*. In comparison with the *CaMV35S* promoter, the *JcUEP* promoter was less effective in most tissues. This is similar to the constitutive *UBQ1* and *UBQ6* promoters from *Arabidopsis* and the *uep1* promoter from oil palm, all of which showed lower activities than the *CaMV35S* promoter (Callis et al. 1990; Masura et al. 2010). We observed that the phenotype of transgenic *Jatropha* produced by the *JcUEP*-driven transgene was the same as that of the *CaMV35S* promoter plants (data not shown). This suggests that the *JcUEP* promoter is readily competent for driving transgene expression for trait modification of *Jatropha*. In addition, unlike the *CaMV35S* promoter cloned from cauliflower mosaic virus, *JcUEP* promoter is an endogenous promoter from this species; thus, it has the advantage of biosafety in transgenic breeding of *Jatropha* (Hull et al. 2000; Potenza et al. 2004).

Another consideration is that overuse of the *CaMV35S* promoter in the same vector increases the probability of gene silencing (Matzke and Matzke 1995; Xiao et al. 2005). In this work, we found that some shoots generated from *CaMV35S:GUS* transformation events turned completely yellow in the screening medium but showed *GUS* staining (data not shown). This indicated that *nptII* did not

function, so the shoots failed to resist kanamycin. One of the reasons for *nptII* silencing in the transformed shoots was probably associated with promoter homology (Park et al. 1996), because pCAMBIA2301 contains two *CaMV35S* promoters, driving both *nptII* and *GUS* expression. Eliminating repeated elements from transgene constructs and moderate transcriptional rates would mitigate the problem (Matzke and Matzke 1995). Therefore, exploring more alternative constitutive promoters to the *CaMV35S* promoter is very important.

In addition, a large variation in *GUS* activity was observed among independent transgenic T0 *Jatropha* plants with both *JcUEP:GUS* and *CaMV35S:GUS* (Table 3). Further experiments on T1 plants are required to confirm the difference in *GUS* expression among different transgenic lines. However, this phenomenon is not uncommon in the transformation of other plants. In an *Arabidopsis UBQ1* and *UBQ6* promoter study, the *GUS* activities varied notably among transgenic lines of tobacco (Callis et al. 1990). A great variation in *GUS* expression was observed between transformants of *Gladiolus* containing the *CaMV35S* promoter (Kamo et al. 2000). The *UBQ1*, *Ubi.U4* and *MtHP* promoters also produced extensive variation in transgenic *Arabidopsis*, *Nicotiana tabacum* and *Medicago truncatula*, respectively (Holtorf et al. 1995; Plesse et al. 2001; Xiao et al. 2005). This phenomenon may result from multiple factors such as the position effect, copy number, rearrangement or repetition of the T-DNA (Hobbs et al. 1990). In this study, we found a large variation in *GUS* activity between line A3 and A9 of *JcUEP:GUS* transgenic plants and among line D7, D12 and D15 of *CaMV35S:GUS* transgenic plants (data not shown), although all these transgenic lines contained single-copy transgene (Fig. 4). Therefore, the copy number seems not to be the main reason causing great variation in transgene expression level among the different transgenic lines. In many transgenic plants, the position effect was the most likely factor (Grec et al. 2003; Peach and Velten 1991; Rooke et al. 2000; Xiao et al. 2005). The T-DNA is integrated into the host genomic DNA randomly, and the expression of the introduced gene could be affected by the adjacent host DNA. Since the use of matrix-associated regions (MAR) flanking the transcription unit can reduce the position effect (Mlynarova et al. 1994, 1995), it is worth trying to use MAR to stabilize transgene expression in *Jatropha* transformation.

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

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