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Isolation and characterization of curcin genes with distinct expression patterns in leaves and seeds of *Jatropha curcas* L.

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ABSTRACT

Jatropha (*Jatropha curcas* L.) is an emerging biofuel plant, of which its seed oil is suitable for biodiesel production. Curcin, a type 1 ribosome inactivating protein, is the major toxic protein found in jatropha seeds. Here we report the cloning and characterization of three curcin genes from *J. curcas* MD44. The *Curcin 1* (*C1*) gene encodes a Type I curcin precursor containing 293 amino acid residues. The *C1* gene is specifically expressed in the endosperm of jatropha seeds at late developmental stage. The *Curcin 2A* (*C2A*) gene encodes a Type II curcin precursor containing 309 amino acid residues and is mainly expressed in young leaves of *J. curcas*. The *Curcin 2B* (*C2B*) gene is another Type II curcin gene that is physically linked to the *C1* gene. However, the expression of the *C2B* gene was not detected in leaves or seeds of jatropha plants growing under normal growth condition. A conserved 227-bp or 228-bp intron was identified or predicted in the 5′ untranslated region of the three curcin genes. A *C1* promoter (−2888 bp to +293 bp) was identified to harbor all *cis*-elements that are required for full promoter activity in jatropha endosperm. The isolation of the three curcin genes and the endosperm-specific *C1* promoter provide useful information and research materials for further functional study of curcin proteins and genetic engineering of *J. curcas*.

1. Introduction

Jatropha (*Jatropha curcas* L.) is a tropical shrub yielding high quality non-edible oil. In recent years, jatropha has become a potential oilseed crop for the production of renewable bioenergy (Maghuly and Laimer, 2013). The kernel of its seed has high crude protein (22–28%) and oil (54–58%) contents (Devappa et al., 2010). Production of jatropha oil could generate similar amount of seed cake. However, jatropha seeds contain toxic and anti-nutritive compounds, such as phorbol esters and curcins (Sabandar et al., 2013). The presence of these compounds in jatropha has resulted in seed cake being unsuitable to be used as animal feed and hence raised safety and environment concerns on jatropha plantation and processing (He et al., 2011; Pradhan et al., 2012).

Ribosome-inactivating proteins (RIPs) are toxic N-glycosidases that depurinate the universally conserved α-sarcin loop of large rRNAs and inactivate the ribosome by blocking its further participation in protein synthesis (Nielsen and Boston, 2001; Sikriwal and Batra, 2010). Based on their overall structure, RIPs are classified into two major groups. Enzymes that consist exclusively of a single polynucleotide adenine glycosylase (PAG) domain are referred to as type 1 RIPs whereas type 2 RIPs are chimeric proteins with PAG domain linked to a C-terminal lectin domain. Curcin is a type 1 RIP that is commonly present in the members of Euphorbiaceae family. Lin et al. (2003a) isolated the first curcin gene that encodes a seed-specific curcin precursor. Previous genome sequencing analysis suggested that *J. curcas* has three curcin genes and two additional curcin-like genes (Sato et al., 2011). However, the sequences of the DNA contigs that contain the curcin or curcin-like genes are not available in public domains or on the website provided (http://www.kazusa.or.jp/jatropha/). Recent gene annotation work identified one Type I curcin (XP012074335), two Type II curcins (XP012074346 and XP012074358) and three curcin-like proteins (XP012074106, XP012074368 and XP012074421) in *J. curcas* with their genomic sequences available in GenBank (Zhang et al., 2014). So

Abbreviation: BAC, bacterial artificial chromosome; *C1*, *Curcin 1*; *C2A*, *Curcin 2A*; *C2B*, *Curcin 2B*; ERE, ethylene responsive element; GUS, β-glucuronidase; *JcActin1*, jatropha actin gene 1; kDa, kilo-Dalton; PAG, polynucleotide adenine glycosylase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends; RIP, ribosome-inactivating protein; SD, standard deviation; SNP, single nucleotide polymorphism; TAIL-PCR, thermal asymmetric interlaced polymerase chain reaction; *Ubi1*, maize ubiquitin gene 1; X-Gluc, 5-bromo-4-chloro-3-indolyl glucuronide; 5′UTR, 5′ untranslated region; 3′UTR, 3′ untranslated region.

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far, more than ten curcin genes and several curcin-like genes have been identified from different jatropha accessions and the predicted amino acid sequences of curcin precursors are available in GenBank. These curcin precursors share at least 86% identity at amino acid level and are classified into two types. Type I curcin precursors have 293 amino acid residues, whereas Type II curcin precursors contain 309 amino acid residues. Each curcin precursor carries a conserved signal peptide containing 42 amino acid residues, which is removed during curcin maturation (Lin et al., 2003a). The mature Type I and Type II curcins are 28 kilo-Dalton (kDa) and 30 kDa, respectively (Lin et al., 2003a; Wei et al., 2005; Qin et al., 2010). Type I curcins were only identified in jatropha seeds (Lin et al., 2010; He et al., 2011; King et al., 2011), whereas Type II curcins were mainly found in jatropha leaves and induced by biotic and abiotic stress (Wei et al., 2005; Qin et al., 2010). The biochemical function of curcins in jatropha is not known. Type I curcins in seeds may serve as storage proteins to provide nutrition during seed germination. The toxic proteins may also prevent animals from consuming jatropha seeds (Stirpe, 2004). Type II curcins in leaves may play a role in defense against biotic and abiotic stress (Wei et al., 2005; Huang et al., 2008; Qin et al., 2010). Besides, curcins were found to demonstrate antitumor activity, and hence they have promising potential in cancer therapy (Lin et al., 2003b; Luo et al., 2006; Mohamed et al., 2014a; Mohamed et al., 2014b; Jaramillo-Quintero et al., 2015). In addition, the promoters of Type I and Type II curcin genes have been isolated by PCR and characterized in transgenic tobacco (Qin et al., 2009a; Qin et al., 2009b). Here we report the isolation of one Type I curcin gene, *Curcin 1* (*C1*), and two Type II curcin genes, *Curcin 2A* (*C2A*) and *Curcin 2B* (*C2B*), from *J. curcas* and the characterization of their distinct expression patterns in leaves and seeds of *Jatropha curcas* L.

2. Material and methods

2.1. Plant materials and growth condition

J. curcas MD44 was used for the experiments. Plants were grown in experimental field in Singapore with temperatures ranging from 32 °C during the day to 26 °C at night with 85% relative humidity and photoperiod of 12 to 13 h.

2.2. Constructs

To make a construct that carries *GFP* reporter gene for promoter analysis, a synthetic *GFP* gene was amplified from pSSZ41 (Kolesnik et al., 2004) and cloned into binary vector pC1300 to generate pCGFP. A 216-bp *C1* terminator was cloned into pCGFP at sites between *Kpn*I and *Eco*RI to generate pCGFPT1. *C1* promoters of different length were amplified by PCR and cloned into pCGFPT1 at the sites between *Hind*III and *Pst*I to generate transcriptional fusion constructs. *C1* promoters were also used to replace the CaMV 35S promoter at the upstream of the intron-containing β-glucuronidase gene (*GUSPlus*) in CAMBIA vector pC1305.1 to create promoter-*GUSPlus* fusion genes.

2.3. Rapid amplification of cDNA ends (RACE) and thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR)

5′ RACE and 3′ RACE were carried out according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The primers for 5′ RACE of *C1* were primer pair GSP1 and AAP, and primer pair GSP1A and AUAP (Table 1). The primers for 3′ RACE of *C1* were primer pair C2RTF2 and AUAP, and primer pair 3′RACEF4 and AUAP (Table 1). The primers for 5′ RACE of *C2A* were primer pair C2RTR2 and AAP, and primer pair 5′RACEG4 and AUAP (Table 1). The primers for 3′ RACE of *C2A* were primer pair CTRTF2 and AUAP, and primer pair 3′ RACEF1 and AUAP (Table 1). TAIL-PCR was performed according to the procedures published previously (Liu and Whittier, 1995). The nested primers were C2AFAR1, C2AFAR2 and C2AFAR3 (Table 1). The arbitrary degenerate primer was AD2 (Table 1).

Table 1
DNA oligo primers used in this study.

Primer	Nucleotide sequence (5′ to 3′)
GSP1	GTCTGCTGGCTTTGAACTTT
GSP1A	CACCCCTAAATCCACATCCT
AAP	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGGGG
AUAP	GGCCACGCGTCGACTAGTAC
C2RTF2	GGGCATCGGCTAGGGAAATA
3′RACEF4	TCTCATCAAACCAAAACTACAAA
C2RTR2	AGAGGTCTCCCCAGTTGTTC
5′RACEG4	TGAATCTTGCTGCCTCTG
CTRTF2	GGGCATCGGCTAGGGAAATA
3′RACEF1	GAGAGGATGTGGATTTAGG
C2AFAR1	CTAAACTCAAACTCAAATCATC
C2AFAR2	CTAACGCTCATCTTAAGCTTAGTA
C2AFAR3	CCATAACCAATGTATGATTTGGTA
AD2	NGTCGASWGANAWGAA
Cantigen F1	GCTGGTTCCACTCCAACTTTAAC
Cantigen R1	TCAGACTTTGTATTTGACTGCATTC
Cantigen R2	CCCAAGCTTAATTATCTAATGCCTGCACCCC
CF2	GATATTTGTGTTTCTTCAT
CR2	TTTGTTGTCCTTTATTTATGCT
C2PF4	CTTCAAGACACTAGTTCAAAAA
C1CDSR	TGATGAGAATGGACAAACTAT
C2ACDSF	CCCAAGTAAAAGGTCTCA
C2TR2EcoRI	CGGAATTCGTATTATTTGGATGGTAGAAAATT
C2AP3PmlF	CGGCACGTGAAAGTTAGAGTTAGGGTATAA
C2APstR	AACTGCAGATTGATTTCACCTGTCCAGTTGTA
CF	TTTACTTCCCCGTTTGCTCA
C1SF	TGCAGTCAATTACAAAGTCTG
C2ASF	AGTAAAAGGTCTCATGGGAGTC
C1SR	TATAGCATTAGCAACAATAATAAT
C2ASR	CATTAGCAACATGTTTGGACAAC
JcActin1-F	TAATGGTCCCTCTGGATGTG
JcActin1-R	AGAAAAGAAAAGAAAAAAGCAGC

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was conducted in accordance with the procedures described previously (Gu et al., 2011). Total RNA extracted from endosperm of developing seeds, leaves at different developmental stages or leaves infected with aphids (*Aphis craccivora*) were used for DNase I treatment and 1st strand cDNA synthesis according to the manufacturer's instructions (Bio-Rad). A standard reaction mixture (15 μl) consisted of 2 μl 1st strand cDNA, 1× SsoFast EvaGreen supermix (Bio-Rad) and 500 nM forward and reverse primers. The PCR reaction consisted of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s. A melting-curve reaction was subsequently performed for 10 s, starting at 65 °C with 0.5 °C increments. The expression of jatropha actin gene 1 (*JcActin1*) was used as the internal control. The average expression levels of *C1* in 6-week-old jatropha endosperm and *C2A* in young leaves were set as "1", respectively. The qRT-PCR experiments were performed in triplicate, and the data are presented as means ± standard deviation (SD). The specific primer pairs for the *C1* and *C2A* genes were C1SF/C1SR and C2ASF/C2ASR, respectively (Table 1). The specific primer pair for the *JcActin1* gene was JcActin1-F/JcActin1-R (Table 1).

2.5. Southern blot analysis

Southern blot analysis was carried out according to the standard procedures (Sambrook and Russell, 2001). Plant genomic DNA was isolated from leaves as described previously (Dellaporta et al., 1983). About 2–5 μg of DNA was digested with restriction enzymes by *Bam*HI, *Eco*RI, *Hind*III or *Xba*I, separated on 0.8% agarose gel and then blotted onto Hybond™-N + nylon membrane (Amersham Biosciences,