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ISOLATION, CLONING AND CHARACTERIZATION OF ACTIN-ENCODING cDNAs FROM *Jatropha curcas* L. IP-2P

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Abstract

Actin is a major component of the plant cytoskeleton, so all cells contain this protein. Actin is expressed constitutively and is involved in basic housekeeping functions required for cell maintenance. Because of this, it has been frequently used as an internal control to normalize changes in gene expressions analysis. Actually, the information of nucleotide sequence of actin gene of *Jatropha curcas* L. population IP-2P from Indonesia is not available yet. The objective of this research was to isolate, clone and characterize cDNA of actin genes of *J. curcas* IP-2P. Three partial actin gene sequences had been successfully isolated by PCR using total cDNA as template, and actin primer designed from conserved region of *Arabidopsis thaliana*. Nucleotide sequence analysis showed that the length of *JcACT* fragment is 610, 534, and 701 bp encoding 203, 177, and 234 amino acids respectively. Local alignment analysis based on mRNA sequences shows that JcACT fragment shares 98% similarity with actin mRNA of *Hevea brasiliensis* and 99% with actin mRNA of *Ricinus communis*. Based on deduced amino acid sequence, JcACT is 100% identical to actins from *Prunus salicina*, *Gossypium hirsutum*, and *Betula luminifera*. Even though these clones of cDNA are not completed yet, they can be used as reference in *J. curcas* L. gene expression analysis.

Keywords: actin, cDNA, cloning, gene isolation, Jatropha curcas L. IP-2P

1. Introduction

Actin is a ubiquitous and highly conserved protein with a molecular mass of approximately 42 kDa, found in all eucaryotic cells. The most conspicuous cytoplasmic actin cytoskeleton in plant cells is essential for a wide range of cellular processes, including establishing and maintaining cell shape and polarity, tip growth, cytoplasmic streaming, organelle movement and repositioning, cell division, and responses to external signals [1–4]. Actin is a multi-functional protein encoded by a large gene family in plants. For example, there are ten genes of the actin gene family in *Arabidopsis*, all of which have been cloned, sequenced, and characterized in detail [5]. At least eight of the actin genes appear to be functional and are strongly expressed at some time and place during plant development [6–8].

The actin genes are all relatively small (i.e. <3 kb) and are individually dispersed in the genome [9]. The typical actin gene has an approximately 100-nucleotide 5‘untranslated region (UTR), a 1200-nucleotide translated region and a 200-nucleotide 3’UTR. Actin contains up to six introns. Soybean actin is composed of 376 amino acids. Coding sequence of these actin genes was interrupted by three small introns 90 bp each [10]. Analysis of the actin gene *act1* from maize and soybean revealed that the intron position inside the actin gene was conserved while the coding sequence varied [11].

Plant stress studies are based more on gene expression. The analysis of gene expression requires sensitive, precise, and reproducible measurements for specific mRNA sequences. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), is at present the most sensitive method for the detection of low abundance mRNA. To avoid bias, qRT-PCR is referred to one internal control gene, which should not fluctuate during treatments. The use of these internal control genes in molecular biology is dependent on the assumption that their expression level remains constant from cell to cell, sample to sample, and treatment to treatment [12]. Actin, together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin,

cyclophilin, elongation factor 1-α (ef1α), ubiquitin, and 18 Svedberg Units (S) rRNA (18S rRNA), are expressed constitutively and are involved in basic housekeeping functions required for cell maintenance. Because of this, they are commonly used as endogenous internal controls to normalize gene expression studies [13].

Until recently, the actin gene has never been isolated from *J. curcas* L. var. IP-2P from Indonesia. Therefore, the objectives of this research were to isolate, clone and characterize the cDNA encoding actin from *J. curcas* L. IP-2P. In this study, we used the actin gene for quantifying RNA levels under aluminum (Al) stress in the expression analysis of transgenic *J. curcas* L. IP-2P carrying *almt* (aluminum-activated malate transporter) gene.

2. Methods

Root and leaf samples of *J. curcas* L. IP-2P were collected and used as plant materials. The frozen root and leaf samples were placed in liquid nitrogen and used for total RNA extraction. The extraction was conducted by RNeasy Plant Mini Kit (QIAGEN) and then the total RNA was treated with RNAse-free DNAse using RNA cleanup Kit (QIAGEN) to remove the contamination of genomic DNA.

The total RNA was then quantified by UV-VIS spectrophotometer (Beckman DU 40) based on absorption at 260 nm. The integrity of total RNA was determined by electrophoresis on 1% agarose gel (FMC, USA) with TAE buffer solution (0.04 M Tris, 0.001 M EDTA-Na_2 $2H_2O$, 0.02 M acetic acid pH 8.5). For electrophoresis, 1 µL total RNA was mixed with 1/6 volume loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and electrophoresed at 100 volt 30 minutes. Visualization of RNA was performed on UV transluminator CS analyzer (Atto Densitograph Software) after stained with ethidium bromide solution (0.5 µg/mL) for 10 minutes, and washing by H_2O and photographed using a digital camera.

Quantified RNA was reversely transcribed into cDNA utilizing ReverTra Ace® qPCR RT Kit (Code No. FSQ-101, Toyobo). The 20 µL-reaction containing 1 µg of total RNA, 2 µL 5x RT buffer, 0.5 µL oligo (dT) primer, 0.5 µL RT enzyme mix (10 U/µL) and nuclease-free water. cDNA synthesis was carried out at 37 °C for 15 minutes, 98 °C for 15 minutes, and 4 °C for 60 minutes. The purity of total cDNA was verified by using PCR with specific primers. The primers of ActF (ATGGCAGATGCCGAGGATAT) and ActR (CAGTTGTGCGACCACTTGCA) designed based on cDNA of β-actin of soybean [10] were used to amplify exon1-exon2 of cDNA of β-actin as a control for the purity of total cDNA. The composition of PCR was: 0.75 µL total cDNA, 1 x buffer taq, 30 mM $MgCl_2$, 3 mM dNTP mix, 15 pmol primer ActF, 15 pmol primer ActR, 0.75 U taq DNA polymerase (Promega), 4% DMSO and nuclease-free water for final volume of 15 µL. The PCR conditions were an initial denaturation at 95 °C for 5 minutes followed by 35 cycles of a denaturing step at 94 °C for 30 seconds, an annealing step at 57 °C for 30 seconds, and an extension step at 72 °C for 1.5 minutes. A final extension step at 72 °C for 5 minutes was included after the 35 cycles.

To isolate fragments of *J. curcas* actin, degenerate PCR primers based on McDowell *et al*. [5] were used. The primer sequences were F1 (PlAc12S): TGYGAYMYGGNACNGGNATGG, F2 (PlAc46S-20): ATGGTNGGNATGGGNCARAA, R1 (PlAc245N-20): GTDATNACYTGNCCRTCNGG and R2 (PlAc284N): ATRTCNACRTCRCAWTCATDAT. The composition of PCR was 1 µL cDNA, 2 µL 10x Ex Taq buffer, 40 mM $MgCl_2$, 4 mM dNTP mix, 20 pmol forward primer, 20 pmol reverse primer, 0.25 U Ex Taq DNA Polymerase (Toyobo), 4% DMSO, and completed to 20 µL by H_2O. Thermal cycling was carried out for 35 cycles of denaturation at 94 °C, for 30 seconds, an annealing step at 56 °C for 30 seconds and an extension step at 72 °C for 1 minutes and 30 seconds, post-PCR at 72 °C for 5 minutes. After amplification, the reaction products were resolved by agarose gel electrophoresis.

The PCR fragments were directly ligated into the pGEM®-T Easy vector (Promega) according to the manufacturer's instruction. The ligation reaction mixtures contained 3 µL of PCR product, 1 µL (10 ng) pGEM®-T Easy, 1 µL (4 U) T4 DNA ligase and 5 µL 2 x rapid buffer ligation. The reaction was incubated at 4 °C overnight and subsequently introduced into DH5α strain of *E. coli* competent cells using heat shock method as described by Suharsono [14].

E. coli DH5α containing recombinant plasmid was selected by using ampicillin and blue-white selection in the solid LB media (10 g/L, 1 bacto-trypton, 5 g/L, yeast extract, 10 g/L, NaCl, pH 7.5, 1.5% bacto agar) containing 100 mg/L ampicillin, 10 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) and 50 mg/L X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White colony grown in the selection media was used as a source of template for PCR to detect the presence of *J. curcas* actin fragment in the transformed bacteria. For this purpose, white colony was picked up by using toothpick, then suspended in 6.5 µL ddH2O and heated at 95 °C in the waterbath for 10 minutes and immediately cooled in the ice for 5 minutes. This suspension was used as the template to amplify the insert with the same composition and condition of PCR as described for isolation of *J. curcas* actin fragments.

To detect colonies bearing the *J. curcas* actin fragment inserted into the plasmid pGEM®-T Easy, we performed

restriction analysis. To excise the insert, Recombinant plasmid DNA was cut by *Eco*R1 (Promega) through mixing 200 ng plasmid DNA, 10 U *Eco*R1, 1x restriction buffer and ddH2O in 20 µL solution. The solution was incubated at 37 °C for 2 hours.

The positive clones were selected for further analysis. Plasmid DNA was isolated from overnight cultures using the QIAprep spin miniprep protocol (Qiagen). Plasmid DNA was sequenced on both strands using T7 and SP6 primers using the Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems Japan Inc.). The actin fragment obtained from *J. curcas* L. IP-2P was designated as *JcACT*. Amino acid sequence was deduced by using translation program of EXPASY (*http://www.expasy.ch/tools/dna.htm*). The *JcACT* nucleotide and deduced amino acid sequence comparisons against the database in The GenBank for homology analysis was performed by BLAST (basic local alignment search tool) (*http://www.ncbi.nlm.nih.gov/BLAST/*) program. The analysis of open reading frame (ORF) was carried out by using BESTORF program (*http://www.softberry/bestorf/htm*). Phylogenetic analyses were conducted using MEGA version 5, and the neighbourjoining (NJ) method was used to build phylogenetic trees [15]. Bootstrap analysis was performed using 1,000 replicates.

3. Results and Discussion

Total RNA of root tips of *J. curcas* had been successfully isolated. Quantification of total RNA by using spectrophotometer showed that there was 250 µg of total RNA per g root tips. This result was enough for the synthesis of total cDNA. The OD260/OD280 ratio of total RNA was 1.9 indicating that isolated total RNA had a very good purity from the protein contaminant [16]. Electrophoresis of total RNA in agarose gel showed two dominant bands (Figure 1). The existence of two bands subunit ribosomal RNA indicates that the total RNA has good integrity. Intact eukaryotic RNA electrophoresed on the agarose gel will have sharp, clear 28S and 18S rRNA bands. Partially degraded RNA will have a smeared appearance and will lack sharp rRNA bands. Isolation of intact RNA is essential for many techniques used in gene expression analysis such as complementary DNA (cDNA) synthesis and Northern analysis.

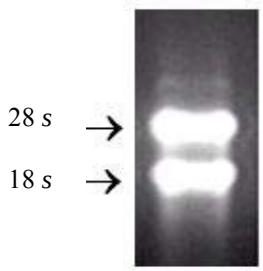

Figure 1. Total RNA from *J. curcas* Root

Total cDNA had been successfully synthesized from total RNA as template by reverse transcription method. By using oligo (dT) primer, only mRNA can be used as a template for cDNA synthesis because it contains poly-A tail. This poly-A tail can form a complementary pair with oligo (dT) primer. PCR by using total cDNA as template and spesific primers for cDNA of exon1-exon2 of β-actin gene resulted one band DNA at 700 bp in size (Figure 2) which were the region of cDNA exon1-exon2. While PCR amplification by using *J. curcas* genomic DNA as a template resulted in more than one band with a bigger size ± 700 bp - 1500 bp because the intron region was included. This result verified the purity of total cDNA without genomic DNA contamination.

Actin fragment from *J. curcas* had been successfully isolated from total cDNA by using a PCR strategy with degenerated primers. The size of the original PCR product are approximately 600--800 bp which are within the expected size for the actins (Figure 3).

Actin fragment from *J. curcas* had been inserted in the middle of *lacZ* gene of pGEM®-T Easy plasmid and this plasmid had been successfully introduced into the *E. coli* strain DH5α. This was demonstrated by the presence of white colonies grown in the selection LB media containing ampicillin, IPTG and X-gal. Only the

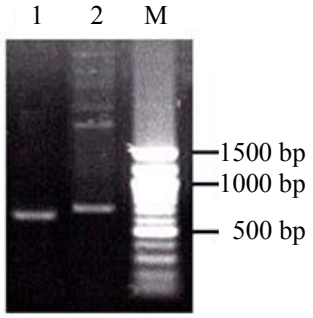

Figure 2. PCR Amplification to Verify the Purity of Total cDNA by Using cDNA (Lane 1) and *J. curcas* Genomic DNA (Lane 2) as a Template with β-actin Primer, M = 1 kb DNA Ladder

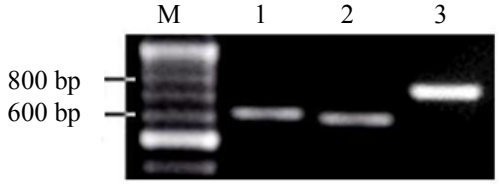

Figure 3. PCR Amplification to Isolate Actin Fragments Using cDNA as a Template and Degenerate Primer for *Actin* Genes, P1Ac12S and P1Ac284N. Lane 1, 2 = Actin Fragments from Root, Lane 3= Actin Fragments from Leaf, M = 1 kb DNA Ladder Markers

E. coli strain DH5α containing recombinant plasmid had a white color. The presence of actin fragment inserted in the recombinant plasmid was confirmed by colony-PCR which resulted 600--800 bp fragment (data not shown).

To reconfirm that actin fragment had been inserted in the pGEM®-T Easy, the recombinant plasmid DNA had been isolated from 34 random white colonies and restriction analysis using *Eco*RI to excise the insert was performed. Restriction digest of recombinant plasmid with *Eco*RI will generate two fragments if there were an insert; one was 3,000 bp fragment DNA corresponding to pGEM®-T Easy vector, and the other was smaller size insert. Three of the colonies in this collection harbored a plasmid carrying a 600−800 bp insert of the actin gene fragment (Figure 4). This result showed that the actin fragment had been inserted into pGEM®-T Easy plasmid. The remaining clones that have smaller insert than 400 bp were not proceeded to a further step.

The nucleotide sequences of those three clones were sequenced. Sequence analysis confirmed that the sequences was the proper reading frame of actin encoding gene and were designated as *JcACT1*, *JcACT2* and *JcACT3*. Sequence data from this article can be found in the GenBank databases under the following accession numbers: HM587793 for *JcACT1*, HM587794 for *JcACT2*, and HM587795 for *JcACT3*. Nucleotide analysis showed that those isolated *JcACT* are partial cds of actin. DNA sequencing of *JcACT1* resulted 610 nucleotides containing 606 bp *ORF* (*open reading frame*). This ORF encodes 203 amino acids. *JcACT2* has 534 nucleotides containing 506 bp ORF which encodes 177 amino acids. *JcACT3* has 701 nucleotides containing 699 bp ORF which encodes 234 amino acids. Local alignment analysis with BLASTn showed that *JcACT1, JcACT2* and *JcACT3* shares 98% similarity with actin mRNA of *Hevea brasiliensis* (Acc. No. GU270586.1), 99% with actin mRNA of *Ricinus communis* (Acc.No.XM002522148.1).

Based on the deduced amino acid sequence of *JcACT*, it is actin protein. A BLASTp search using *JcACT* protein sequences as the query sequence revealed that the *J. curcas* actin protein shares 100% identity with the actins from other species, especially with *Prunus salicina* (ABU68265.1), *Gossypium hirsutum* (AAP73458.1), and *Betula luminifera* (ACJ38662.1).

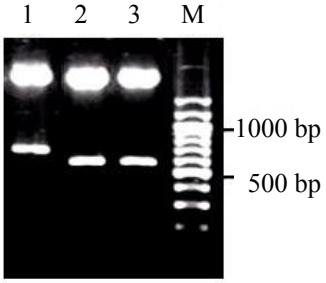

Figure 4. Restriction Analysis of Three Positive Recombinant Clones with *Eco*RI, 700 bp (1), 600 bp (2,3), M = 1 kb DNA Ladder Marker

Those results were supported by the phylogenetic analysis of *JcACT* protein sequences using the full sequences of fifteen actin proteins. The degree of relatedness and similarities between individuals can be seen from a phylogenetic tree. The phylogenetic dendrogram constructed by the neighbor-joining (NJ) method formed three well-defined three separate groups. As indicated in Figure 5, the first group was dicotyledonous-woody plants, which included *Jatropha* and *H. brasiliensis*, which belongs to the same family, *Euphorbiaceae*. The second group was monocotyledonous plants, and the third group was dicotyledon-herbaceous plants. In Figure 5, the phylogenetic tree shows the relationships among three JcACTs based upon similarities and differences in their protein sequences. *JcACT1* and *JcACT2* are more closely related to each other than to *JcACT3*, because *JcACT1* and *JcACT2* were isolated from root samples while *JcACT3* was isolated from leaf. GenBank accession numbers for those 15 sequences are as follows: *Populus trichocarpa* (XP 002331880.1), *Melastoma malabathricum* (BAJ07306.1), *Litchi chinensis* (ADV17460.1), *B. luminifera* (ACJ38662.1), *G. hirsutum* (AAP73458.1), *P. salicina* (ABU68265.1), *H. brasiliensis* (ADB27919.1), *Oryza sativa Japonica* (NP001051822.1), *Zea mays* (NP 001150248), *Sorghum bicolor* (XP 002463566.1), *Glycine max* (ACU17933.1),

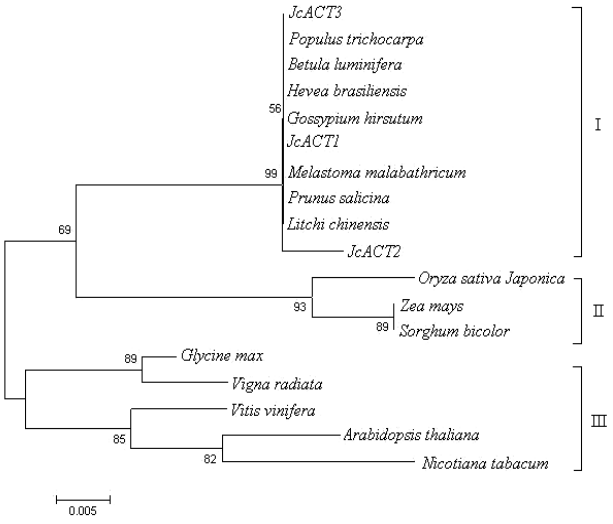

Figure 5. Phylogenetic Tree Describes Relationship among *Jatropha* Actin with Another Actins. Tree Built with the Neighbor-joining Method, Formed Three Well-defined Three Separate Groups: I = Dicotyledonous-woody Plants, II = Monocotyledonous Plants, III = Dicotyledon-herbaceous Plants. The Bootstrap Values Shown

Vigna radiata (AAF31643.1), *Vitis vinifera* (CBI37599.3), *A. thaliana* (NP 196543.1), *Nicotiana tabacum* (ACH69153.1).

Alignment analysis of *JcACT* amino acid sequences with the amino acid sequences of 15 plant actin, allowed the identification of 3 conserved region which located in position 53 to 189, position 191 to 220, and 222 to 250.

These actin cDNA nucleotide sequences were the first actin information from *J. curcas* L. var. IP-2P from Indonesia reported. These fragments are very important for primer designing in the quantitative gene expression analysis using qRT-PCR. In our laboratory we have been interested in the plant mechanism of Al tolerance. The results reported here open the way to further research in the future.

4. Conclusion

From our results, it can be concluded that three cDNA of actin genes from *J. curcas* L. IP-2P had been isolated, cloned and characterized. Those cDNA clones which were 610, 534, and 701 nucleotides in length, have similarity with mRNA *ACT* from *H. brasiliensis* and mRNA *ACT* from *R. communis.* Eventhough these clones of cDNA are not completed yet, they can be used as reference genes in qRT-PCR analysis.

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