

Expression of Six Chloroplast Genes in *Jatropha curcas* Callus under Light and Dark Conditions

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ABSTRACT

The induction of genes encoded in the open reading frames (ORFs) of chloroplast genomes have been posited to be influenced by ambient light condition. The current study focused on determining which of the six ORFs, encoding the genes *ycf 1*, *ycf 2*, *psbD* (photosystem II), *rbcl* (Rubisco), *matK* (Maturase K) and *rpoC1* (RNA polymerase) were influenced by light. Characterization of gene expression at the whole plant level and callus stage facilitates the identification of transcripts which are differentially regulated under these environmental conditions. Specificity of these primers was tested against genomic DNA and total RNA. Transcripts of six targeted genes were detected in all three replicates of the green and white callus under light and dark conditions, except for *ycf 2* gene in green callus under light. The result showed that a partial transcript of the gene *ycf 2* located on the *J. curcas* chloroplast genome was not detectable using reverse transcription PCR. This finding was then validated using quantitative real-time PCR. The gene was suspected to be post-transcriptionally modified. The transcripts of the remaining five ORFs could be detected using quantitative real-time PCR. Specific transcripts can be identified for application as biomarkers for selection of callus for plantlet regeneration.

Keywords: Chloroplast DNA, Gene Expression, *Jatropha curcas*, Post-Transcriptional Modification

1. Introduction

Jatropha curcas is a drought-resistant oil plant which belongs to the family Euphorbiaceae and widely distributed in tropical and subtropical areas, especially in Central and South America, Africa, India and Southeast Asia. The development of *J. curcas* as a high-yielding and efficient new biofuel source is still at a relatively early stage. Some tactical strategies such as molecular characterization of *J. curcas* will facilitate the development of this important oilseed crop [1]. The complete chloroplast DNA (cpDNA) sequence of *J. curcas* is now available (Accession number FJ695500), however there is no data pertaining for the expression of cpDNA open reading frames (ORFs).

The cultivation of *J. curcas* encounters challenges such as disease problems and climate lead to reduced plant productivity. Abiotic stress resulting from abnormal light conditions is one of the major reasons for decline in plant productivity. According to [2], the expression patterns of different genes need to be identified and studied for up-regulation or silencing as part of the transgenic approach to *Jatropha* improvement. Integration of gene analysis

with gene discovery and modelling of genetic networks will facilitate a comprehensive understanding of stress tolerance, permit the development of useful and effective molecular markers, and identify candidate genes for genetic transformation and engineering [3].

In order to carry out a preliminary study on the differential induction of chloroplast genes under different light conditions, a total of six transcripts representing the genes *ycf 1*, *ycf 2*, *psbD* (photosystem II), *rbcl* (Rubisco), *rpoC 1* (RNA polymerase) and *matK* (Maturase K) were targeted. The approach which was undertaken involved the design and application of primers to target specific transcripts which have been conjectured to be influenced by light and dark conditions. The main objectives for this study were to identify the chloroplast genes which are induced under conditions of light and complete darkness in *J. curcas* *in vivo* and *in vitro* and to validate expression of genes using real-time PCR.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Two week old of green and white callus cultured under

the following conditions: 1) green callus under light and 2) 24 h darkness, 3) white callus under light and 4) 24 h darkness were used as experimental materials. Three replicates of each green and white callus for each condition were maintained aseptically on half strength MS basal medium [4]. Leaves of *J. curcas* from the germinated plant grown in a pot were used as control.

2.2. Primer Design

Specific primer pairs to characterize gene expression were designed using Primer 3 software. For the reverse transcription PCR, primers were designed based on the intronless chloroplast DNA genome, which is available at the GenBank (FJ695500). In addition, another set of primers were designed for real-time PCR assay. These primers were designed based on the sequence results obtained after reverse transcription PCR.

2.3. DNA Extraction

DNA was extracted from a fully expanded leaf of *J. curcas* using the 2% CTAB method as previously described in [5] with few modifications. PCR was performed on genomic DNA in order to test for the specificity of primers.

2.4. RNA Extraction, Reverse Transcription and PCR Analyses

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Fermentas). Reverse transcription PCR amplification with specific primers as shown in **Table 1** was done using standard protocols [6] and the results were applied to determine chloroplast

gene induction in the callus and control plant. All the amplifications were carried out in 20 µl reaction volume containing 50 ng of template DNA, 1.5 mM MgCl₂, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl), 1 U *taq* DNA polymerase (Fermentas), 5 µM of primer, 0.2 mM of dNTP mix in a thermocycler (Eppendorf, Germany). The PCR conditions for amplification were 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, annealing at 56°C for (primers KYCF2, YCB, YCFD and PS2D2) and 58°C for (primers YCF1, YCF2, MATK, RBCL and RPOC1) for 40 s, respectively, 72°C for 2 min, with a final extension at 72°C for 10 min.

2.5. DNA Sequencing and Data Analyses

PCR products corresponding to the expected sizes were extracted from the agarose gel using QIAGEN Gel Extraction Kit according to the manufacturer's protocol. Approximately 40 ng/µl extracted PCR products, with 10 pmole/µl of the specific primer, were sent for sequencing at 1st Base Malaysia using the BigDye[®] Terminator v3.1 cycle sequencing kit. Nucleotide sequences were analyzed and compared with the GenBank database of the National Center for Biotechnology Information (NCBI) for the similarity using the Basic Local Alignment Search Tool (BLASTn) online software [7]. The degree of similarity was determined in terms of the E value.

2.6. Quantitative Real-Time PCR

The genes which were confirmed to be not transcribed by the reverse transcription PCR were then verified using quantitative real-time PCR.

In real-time PCR assay, the YCFD2 primers used for the amplification of the 188 bp fragment of part of *ycf2* gene were:

Table 1. Specific primers for the PCR amplification of the chloroplast genes.

Primer Name	ORFs	Primers' Sequence	Ta (°C)	Product (bp)
KYCF2	<i>ycf2</i>	F: TGCAAAGAATCCTCGACGTG R: AACTCGACGATCCTGTTTGG	56	~1600
YCB	<i>ycf2</i>	F: CATGGTTATGGACCCGAATC R: AAATGCCCGGGAGTTCCTCT	56	~1500
YCFD	<i>ycf2</i>	F: GTTCATCCTTCGGAACCAGA R: GACCCCGAATTTGGAGTAT	56	~1400
PS2D2	<i>psbD</i>	F: GATATTATGGATGATTGGTTACGG R: GTTCCACGGGGTAGAACCT	56	~1000
YCF1	<i>ycf1</i>	F: ATCGAGCATTTCCTTTTT R: TTCAAATTCCTCGAATGGTC	58	~850
YCF2	<i>ycf2</i>	F: AAGAGCCGGGAGCAATTTAT R: CGATAGGGCCGATTGGTAA	58	~930
RBCL	<i>rbcl</i>	F: GACAACTGTGTGGACCGATG R: CCAAAGATCTCGGTCAGAGC	58	~990
RPOC1	<i>rpoC1</i>	F: CGGATGTCCCTGCATAACTT R: CTAAGGAGCCGGATGAGAG	58	~990
MATK	<i>matK</i>	F: CTGCGCGAAATAGAGGAAAC R: CCCTTCGCTATTGGATGAAA	58	~970

F: 5'-GAATTCCATTGGACCCAGAA-3'

R: 5'-GCGAACCCAGTTAGATTCA-3'

The RPOC1b primers for the 178 bp fragment of the *rpoC 1* gene were:

F: 5'-CCCGAGTTGAGACCAATCAT-3'

R: 5'-CCACGGCTTCTTGTACCAAT-3'.

Gene expression was analyzed by real time quantitative PCR using the SYBR Green system in an iQ5 Real-Time PCR detection system (Bio-Rad). Real-time PCR was carried out in 50 μ l reaction volume which consisted 25 μ l iQ SYBR Green Supermix (Bio-Rad), 1 μ l of 0.5 μ M of each forward and reverse primers, 3 μ l of RNA and 20 μ l of nuclease free water. PCR reactions were performed under the following thermal cycling conditions: 1 min at 50°C, 5 min at 95°C, 50 cycles of 10 s at 95°C and 30 s at 58°C. Results were confirmed by resolving the product on a 1.5% agarose gel.

3. Results and Discussion

3.1. DNA and RNA Extraction

Using the modified CTAB protocol, the concentration of DNA obtained from *J. curcas* leaves ranged from 16 - 30 μ g/ml. The A_{260}/A_{280} ratio was greater than 1.8, indicating DNA purity. The modified CTAB DNA extraction method was effective in isolating DNA, which could be used for testing the specificity of primers using PCR. The CTAB protocol for DNA extraction worked well for extracting high quality DNA from leaf samples. The CTAB protocol is a rapid and technically easy method for preparing nucleic acids that can be amplified using PCR. This method has been widely applied on various highly consistence of secondary metabolites and lipid plants, such as *Targetes minuta* L. [8], oil palm [9] and *J. curcas* [10].

The RNeasy Plant Mini Kit (Qiagen) was able to extract high quality RNA from *J. curcas* callus. The integrity of RNA was judged by the sharpness of ribosomal RNA bands visualized on 1.5% agarose gel. For all tested RNA samples, distinct 25S and 18S rRNA bands were observed in agarose gel. The A_{260}/A_{230} in the range 1.9 - 2.0 indicated the purity of RNA was suitable for downstream applications. The high quality of RNA was isolated from callus using RNeasy Plant Mini Kit (Qiagen). The RNeasy extraction procedure utilizes a minimum number of reagents and steps, thus reducing the handling time and minimizing the risk of RNase contamination.

3.2. PCR Amplification

In this study, four different primers which amplified specific locus of *ycf 2* gene in *J. curcas* chloroplast genome (Figure 1) were tested. These transcripts accumulated in

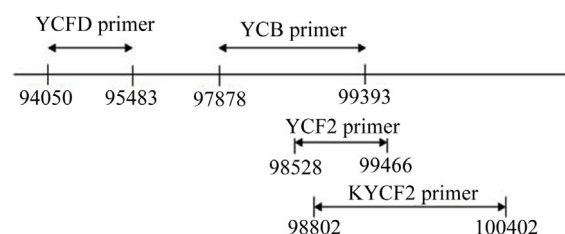


Figure 1. Amplification of *ycf 2* gene targeted at different regions in *J. curcas* chloroplast genome (FJ695500).

green and white callus under light and darkness when amplified with YCB, YCF2 and KYCF2 primers. However, reverse transcription PCR did not detect gene amplified by YCFD primer for the green callus under light treatment and no PCR product was detected. Amplicons with the product size of 1433 bp were visualized from the PCR products of green callus and white callus in dark and white callus under light (Figure 2). It was predicted that part of the *ycf 2* transcript located at position of 94050 to 95483 of *J. curcas* chloroplast genome (FJ695500) was not transcribed in green callus under light condition and it was suspected to be post-transcriptionally modified. Higher eukaryotes respond to environmental signals by regulating their genes. Specifically, gene expression is controlled at two levels. First, transcription is controlled by limiting the amount of mRNA that is produced from a particular gene. The second level of control is through post-transcriptional events that regulate the translation of mRNA into proteins. Even after a protein is translated, post-translational modifications can affect its activity [11].

The *ycf 1* gene transcripts were found in both green and white callus in both light and dark conditions. The results indicated the transcripts from *ycf 1* did accumulate in all the callus treated with differential light conditions and the *ycf 1* gene is suspected to be not related to photosynthesis. Although the function of the *ycf 1* gene has not been ascertained, its presence in a non-photosynthetic plant named *Epifagus virginiana* and experiments in tobacco indicate that it may have function which is probably not related to photosynthesis [12].

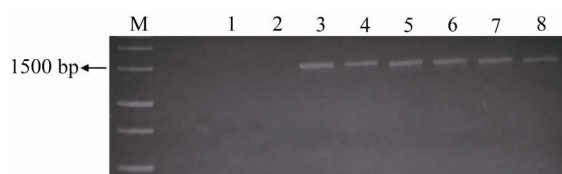


Figure 2. Electrophoresis of PCR products of cDNA amplified by YCFD primer on a 1.5% agarose gel. Lane M: 1 kb O' GeneRuler™ ladder (Fermentas); Lane 1-2: green callus under light; Lane 3-4: green callus in dark; Lane 5-6: white callus under light; Lane 7-8: white callus in dark.

PCR amplification of the *rpoC1* gene yielded 945 bp fragments for all three replicates of each treatment of callus cDNA and leaf under daily natural photoperiod. As shown in **Figure 3**, the cDNA under all treatments mentioned were successfully amplified by the reference gene. This reference gene, encoded the β' subunit of RNA polymerase, was expressed in the treated callus and leaf at all times. The *rpoB* and *rpoC* genes are house-keeping genes that can be utilized in plant transcriptomics [13]. The *psbD* gene, which is a component of photosystem II and responsible for photosynthetic, is not affected by the light and dark treatment in this study. Both the green and white callus treated under light and dark were amplified by the *psbD* specific gene primers (PS2D2 primers). The *matK* was chosen as its region is universally present in land plants and only few exceptions of a secondary loss or reorganisations are known to date [14]. In addition, the *rbcl* gene was chosen as one of the reference gene due to its characteristic of multicopy chloroplast sequence and it is highly conserved sequence in plants [15].

3.3. Nucleotide Sequence Alignments and Database Searches

To further confirm the presence of specific chloroplast genes in callus, the amplified products were sent for nucleotide sequencing. The respective sequences obtained were assembled using DNASTAR SeqManII™ software and then compared with the GenBank database of the NCBI using the BLASTn software. From BLASTn, the sequences obtained from the amplification of genomic DNA leaf and the amplification from cDNA callus were compared. Results showed the sequences achieved high score and expectation (E) value near zero when compared against the *J. curcas* chloroplast genome (FJ695500). These data indicated the sequences resulted from sequencing had high similarity and there was no discernable polymorphism in these genes.

3.4. Real-Time PCR

The regulation of *ycf 2* gene transcripts was validated using the quantitative real-time PCR. From the amplification plot shown in **Figure 4(a)**, the red plot in the amplification curve indicated there was no amplification for green callus under light. The result was confirmed to be similar to the reverse transcription PCR, whereas the gene was not transcribed in the green callus under light. To further verify the *ycf 2* gene amplified by YCFD primers, real-time PCR was carried out to detect the part of *ycf 2* chloroplast gene induction under differential light condition. In the real-time assay, only YCFD primers and RPOC1 primers were performed on the callus cDNA. They were chosen due to the validation of *ycf 2* gene

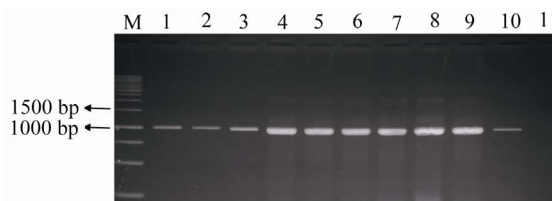


Figure 3. Electrophoresis of PCR products of cDNA amplified by RPOC1 reference primer on a 1.5% agarose gel. Lane M: 1 kb O' GeneRuler™ ladder (Fermentas); Lane 1- 3: green callus under light; Lane 4-6: green callus in dark; Lane 7-9: white callus in dark; Lane 10: leaf (control); Lane 11: negative control.

primed by YCFD while *rpoC 1* was chosen as the house-keeping gene.

As mentioned earlier, failure of transcript detection in part of gene may due to post-transcriptional modification. The chloroplast genome differs in many respects from the nuclear genome. While chloroplast genes are regulated at the transcriptional level, post-transcriptional gene regulation is predominant. As might be expected, light plays an important role in the regulation of many chloroplast genes. According to [16], RNA processing, degradation, and translation are the predominant levels of gene regulation in the chloroplast genome. Post-transcriptional gene regulation is a complex, multistage phenomenon that helps to fine tune the levels of active proteins in the cell.

Primary translation products often undergo a variety of modification reactions, involving the addition of chemical groups which are attached covalently to the polypeptide chain at the translational and post-translational levels. Study carried out by [17] stated plants' subsistence depends on their ability to rapidly regulate gene expression in order to adapt their physiology to their environment. Post-transcriptional regulation of gene expression plays an important role in how plants respond to abiotic stresses such as light intensity, temperature and the availability of water and essential nutrients. In addition, [18] also carried out a study on chloroplast *ycf 2* gene expressions in stressed plants. The study focused on the fruit development in order to verify if *ycf 2* may have a specialized function in non-photosynthetic tissue during seed maturation.

The candidate reference gene *rpoC 1* was used to amplify against the cDNA. **Figure 5(a)** demonstrated the efficiency and sensitivity in the amplification of reference gene. Five steep curves were observed in the amplification profile and this suggested that the reference gene was expressed in all samples under different treatments including the leaf. In addition, in order to confirm the presence of the specific PCR product with product size of approximately 170 bp, the real-time PCR pro-

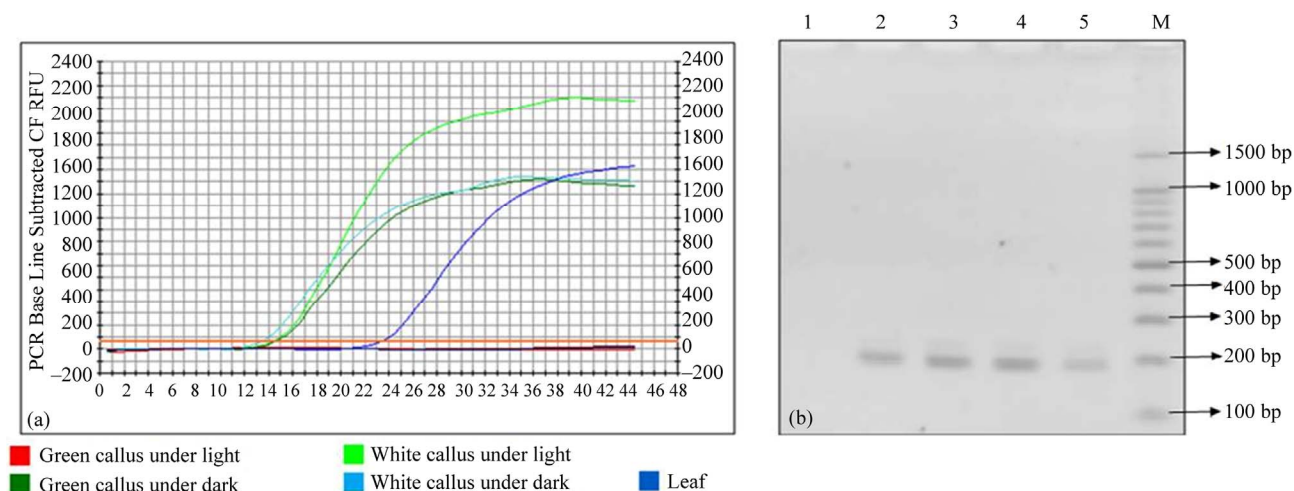


Figure 4. Real-time assay. (a) Real-time PCR amplification plot of cDNA from four treatments of *J. curcas* callus amplified by YCFD2 primer using SYBR Green dye. (b) Electrophoresis of real-time PCR products with approximately 180 bp on 1.5% agarose gel. cDNA was used as template and amplified against YCFD. Lane 1: green callus under light; Lane 2: white callus under light; Lane 3: green callus in dark, Lane 4: white callus in dark; Lane 5: leaf; Lane M: 100 bp ladder (Promega).

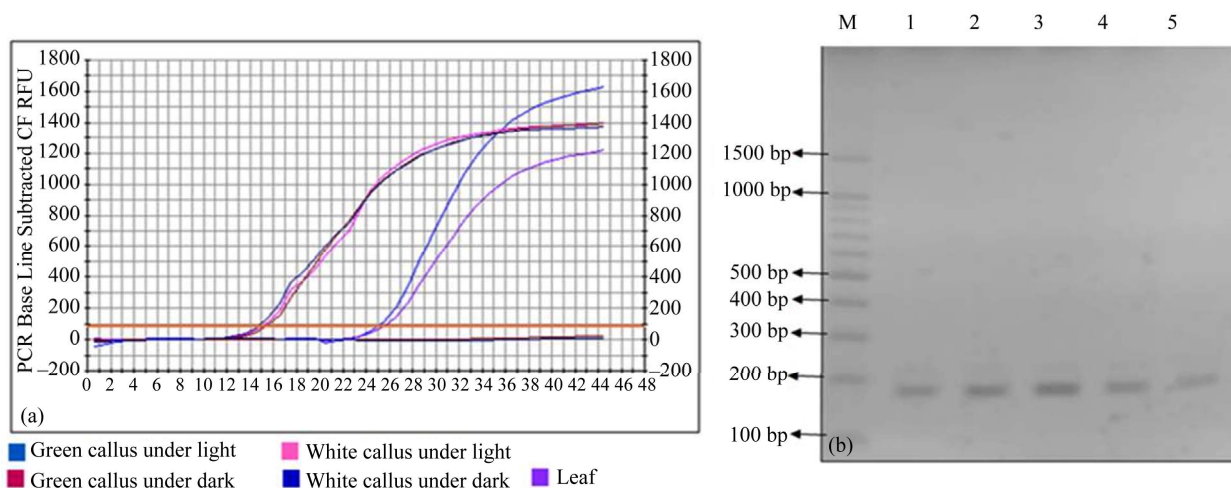


Figure 5. Real-time assay. (a) Real-time PCR amplification plot of cDNA from four treatments of *J. curcas* callus amplified by reference primer RPOC1b, using SYBR green dye. (b) Electrophoresis of real-time PCR products with approximately 170 bp on 1.5% agarose gel. cDNA was used as template and amplified against candidate reference gene *rpoC 1*. Lane M: 100 bp ladder (Promega); Lane 1: green callus under light; Lane 2: white callus under light; Lane 3: green callus in dark; Lane 4: white callus in dark; Lane 5: leaf.

ducts were resolved on 1.5% agarose gel (Figure 5(b)). Under the PCR conditions tested, there was amplification in all the cDNA under four different treatments and leaf against the reference gene *rpoC 1*.

As illustrated in the melting curve in Figure 6, small variations in the T_m indicated a different pattern of amplification. For Figure 6(a), there were four peaks (each represented by green callus under dark, white callus under light and dark, and leaf) presented in the melting curve with T_m of 82.5°C. Five peaks (each represented by green callus under light and dark, white callus under light and dark, and leaf) with T_m in the range of 80°C to

81°C, were apparent in the melting curve shown in Figure 6(b). Other than the significant single sharp peak, there was an extra single low peak produced. This low peak was suspected to be generated by non-specific amplification.

4. Conclusions

Information pertaining to the abiotic stress response of *J. curcas*, with specific reference to light stress will provide a basis for selection of callus tissue for regeneration in plant tissue culture systems. This investigation provided an insight into the expression of the *ycf2* gene and estab-

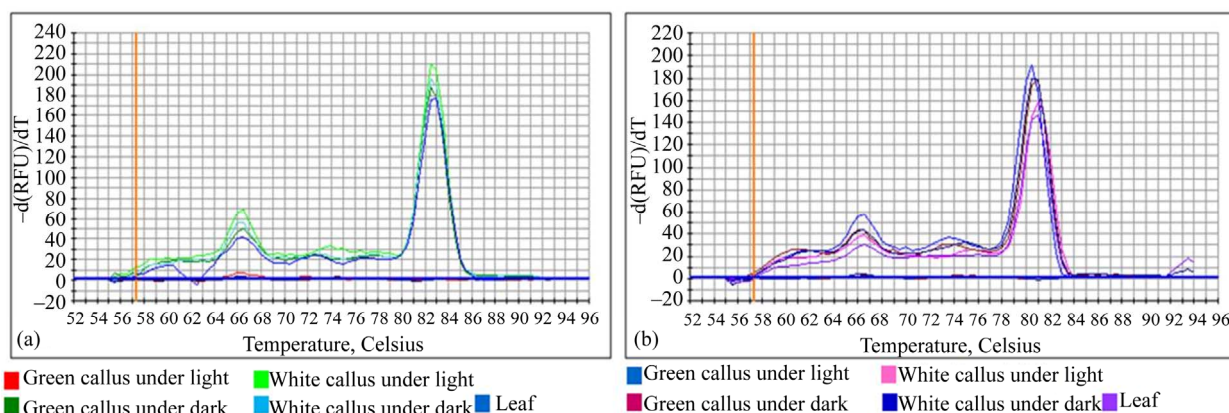


Figure 6. Melting curve profile for real-time PCR amplification of cDNA from four treatments of *J. curcas* callus. (a) cDNA primed with YCFD2. (b) cDNA primed with RPOC1b.

lished a foundation for further analysis of this little studied gene.

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