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LETTER ARTICLE

Identification and Characterization of Genes in the Curcuminoid Pathway of *Curcuma zedoaria* Roscoe

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Abstract: ***Background***: Curcuminoid genes have an important role in the biosynthesis of curcumin, a valuable bioactive compound, in *Curcuma* species. However, there have not been any reports of these genes in *Curcuma zedoaria*.

Objective: The present work reports on the isolation of genes encoding enzymes in curcuminoid metabolic pathway and their expression in *C. zedoaria*.

Method: The primers were designed from untranslation regions of *DCS*, *CURS1*, *CURS2* and *CURS3* genes which are involved in curcuminoid biosynthesis in *C. longa* to isolate the corresponding full-length genes in *C. zedoaria*. RT-PCR amplification and HPLC analysis are used to estimate the expression of genes and biosynthesis of curcumin in both rhizome and callus.

Results: The results showed that all four genes from *C. zedoaria* (named *CzDCS*, *CzCURS1*, *CzCURS2* and *CzCURS3*) and *C. longa* have a high identity (approximately 99%) and lengths of genes from *C. zedoaria* are 1382, 1240, 1288 and 1265 nu, respectively. *CzCURS1*, *2* and *3* genes have one intron while *CzDCS* has two introns. RT-PCR amplification indicated that curcuminoid genes expressed mRNA in rhizome and callus of *C. zedoaria*. Curcumin, a major component of curcuminoids, was also found in callus by HPLC analysis.

Conclusion: The sequence information of *DCS* and *CURS1-3* genes in *C. zedoaria* will be very valuable for a subsequent study on the effects of elicitors on the transcription of genes involved in curcuminoid biosynthesis pathway.

Keywords: *Curcuma zedoaria*, callus, curcumin, curcumin synthase genes, curcuminoid metabolic pathway, diketide-CoA synthase gene.

1. INTRODUCTION

Curcuma zedoaria, belonging to the ginger family (Zingiberaceae), is an indigenous medicinal herbaceous in Bangladesh, Sri Lanka and India, and is also widely cultivated in China, Japan, Brazil, Nepal and Thailand [1], and Vietnam [2].

Curcuminoids, a mixture of curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are found in the rhizome of *C. zedoaria* [1], *C. longa* [3], *C. caesia* [4], *C. aromatica* [5], and *C. amada* [6]. The biosynthesis pathway of curcuminoids in *C. longa* was reported by Katsuyama *et al.* [3, 7]. According to these studies, the *DCS*, *CURS1*, *CURS2* and *CURS3* genes are involved in metabolism, which starts from phenylalanine to form curcuminoid compounds (Fig. **1**). Curcumin (bis-α-β unsaturated β-diketones), a major component of curcuminoids, is invariably present in all *Curcuma* species and is well-known to have therapeutic effects on a variety of human diseases, and the cancer preventive activity of curcumin is being intensively studied all over the world [8]. Previous reports indicated that curcumin has various pharmacological properties and shows anti-inflammatory, anti-oxidant, anti-bacterial and anti-cancer activities [9, 10].

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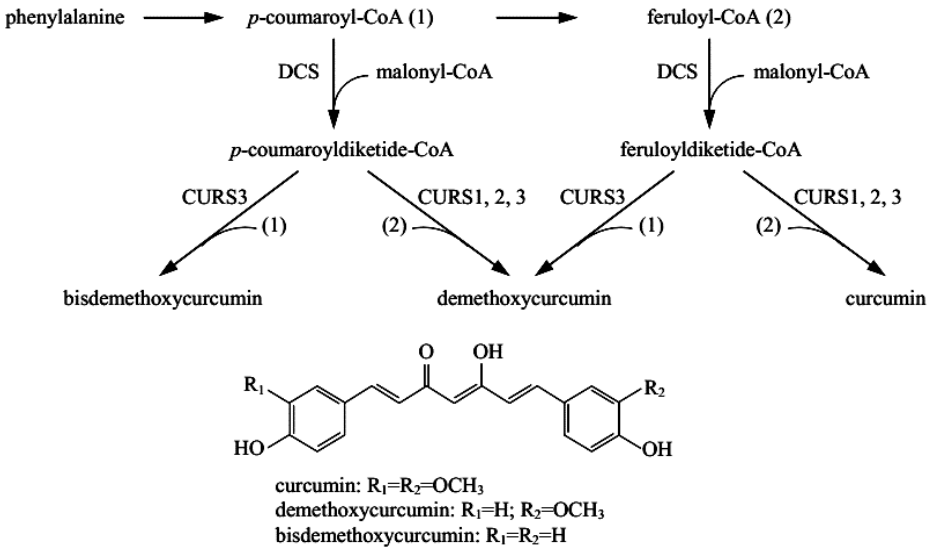

Fig. (1). The biosynthesis pathway of curcuminoids in *C. longa* (Katsuyama *et al.* 2009a,b). *DCS*: diketide-CoA synthase gene; *CURS1*, *2*, and *3*: curcumin synthase 1, 2 and 3 genes.

As is already known, medicinal plant cell cultures for the production of valuable secondary metabolites are promising as potential alternative sources for the production of important pharmaceuticals [11]. Based on a well-known observation that certain elicitors can induce a defense response in plants, thereby upregulating secondary metabolite production [12-14], many studies have attempted to apply elicitors (*e.g.* salicylic acid, methyl jasmonate, yeast extract...) to plant cell cultures for the production of high-value compounds used as pharmaceuticals [15-17]. Several reports also showed that wherever there was an increase in the production of the target bioactive compound through the effects of elicitors, transcription of genes involved in biosynthesis also increased [18, 19].

Naturally, we would like to extend this method to *C. zedoaria* to improve the production of curcuminoids and examine the changes in transcription of involved genes. However, prior to this study, genes which metabolize curcuminoids in *C. zedoaria* have never been reported, though homologous genes in some of its relatives, such as *C. longa* have been well characterized [20] and their sequences are available on NCBI. As a preliminary step for the investigation of the effects of elicitors on the production of curcuminoids in cultured *C. zedoaria* cells, this study aims at identifying genes related to the biosynthesis of curcuminoids and some their characteristics in *C. zedoaria*. To our knowledge, this is the first report on the identification of genes related to curcuminoid biosynthesis in *C. zedoaria*.

2. MATERIALS AND METHODS

2.1. Callus Culture

Leaf-base explants (approx. 0.5 × 0.5 cm) of *in vitro* zedoary plants were transferred to MS (Murashige and Skoog) medium [21] containing 2% (w/v) sucrose, 0.8% (w/v) agar, supplemented with 3 mg/L BAP (benzylamino purine) and 3 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) to induce callus formation [22]. The calli were subcultured on fresh medium every 4 weeks. The culture was incubated at 25±2°C with a photoperiod of 10 h daylight at an intensity of approx. 2,000 lux.

2.2. PCR Amplification

Total DNA of zedoary was extracted from young leaves of *in vitro* plants by the CTAB (cetyltrimethyl-ammonium bromide) method according to Babu *et al.* [23] with slight modification.

The CDS (coding DNA sequence) of putative *CzDCS* (*C. zedoaria DCS*), *CzCURS1*, *CzCURS2* and *CzCURS3* genes was amplified from total DNA by PCR with primers shown in Table **1**. The primers were designed based on the 5'- and 3'-untranslated regions of *DCS*, *CURS1*, *CURS2* and *CURS3* genes from *C. longa* (AB495006.1, AB495007.1, AB506762.1 and AB506763.1, respectively) to ensure specificity of the PCR products due to very high sequence identity among these genes. To obtain the regions flanking CDS sequences of genes involved in curcuminoid pathway of *C. longa*, the mRNA sequence of these genes was BLAST against the EST (expressed sequence tag) database of *C. longa*, and hits with significant E-value score were selected and used with the corresponding CDS sequences to create contigs of full-length mRNA. The supposed full-length mRNA of *DCS*, *CURS1*, *CURS2* and *CURS3* in *C. longa* was used as template for designing primers to amplify corresponding genes in *C. zedoaria*.

PCR components were as follows: 50 ng template DNA, 10 pmol each primer, 6 µL 2× GoTaq® Green Master Mix (Promega), and ddH_2O in a final volume of 12 µL. The amplification was performed in thermocycler (Veriti 96 Well Thermal Cycler, ABI) with a genomic denaturation of 95°C for 15 min; followed by 30 cycles of 95°C for 30 s, 53ºC for 30 s, and 72ºC for 1.5 min; and a final extension of 72ºC for 10 min.

Table 1. Primers used for PCR amplification of the coding DNA sequences of curcuminoid genes in *C. zedoaria*.

Genes	Primers	Nucleotide Sequences (5'- 3')	Putative Length (nu)
CzDCS	DCS-F	GTCGTTTCTGTGACCTTCTC	~ 1400
	DCS-R	CTTTTGGATGCAGACTGGAACA	
CzCURS1	CURS1-F	CTGCGACTGCGAGAAGAAGC	~ 1250
	CURS1-R	CAGATAGACAGCCATACAAACC	
CzCURS2	CURS2-F	GCACGCGTTTTCTTGCTAATC	~ 1300
	CURS2-R	GATCGTGTTCATAATTCACTGG	
CzCURS3	CURS3-F	CTAGCTAGCTGCAATTCGTT	~ 1250
	CURS3-R	GTGCTAGCTTAGCTTGACGTA	

Table 2. Primers used for RT-PCR amplification of the specific regions of curcuminoid genes in *C. zedoaria*.

Genes	Primers	Nucleotide Sequences (5'- 3')	Length of Indicators (nu)	Annealing Temperature (°C)
CzDCS	ID-F	TGCTCCGAGGTCACCGTGC	272	55
	ID-R	GGTCAGCCCAATTTCGCGG		
CzCURS1	IC1-F	CCGCTGGAAGGAATTGAAAAA	286	55
	IC1-R	GAGCTTGTCCGGGCTCAGCTG		
CzCURS2	IC2-F	CCACCTCCGCGAGGTGGGGCT	211	55
	IC2-R	GCGGTGGCCAGCTTGCTCTGT		
CzCURS3	IC3-F	CACCTGAGGGAAATCGGCTGG	202	50
	IC3-R	GCGAGCTTCCCCTGTTCCAGC		

2.3. Gene Cloning and Annotation

PCR products were purified by GeneJET Gel Extraction Kit (Thermo Scientific) according to manufacturer's instructions and then inserted in pGEM®T-Easy vector (Promega). A ligation reaction (10 µL) including 50 ng of vector, 70 ng of PCR product, 3 unit of T4 DNA ligase (Promega), and 1 µL of 10× ligation buffer was incubated at 4°C overnight. Recombinant pGEM®T-Easy vector was then transformed in *E. coli* TOP10 (Invitrogen) by heat-shock method. Nucleotide sequences of PCR products were determined by the fluorescent dideoxyterminator method in ABI 3130 system.

To annotate newly obtained genes from *C. zedoaria*, several methods were employed to ensure high accuracy such as alignment against the corresponding mRNA sequence from *C. lon*ga, blastx and gene prediction by FGENES software.

2.4. Phylogenetic Analysis

Phylogenetic analysis of gene sequences was also employed for annotation of new genes from *C. zedoaria*. The CDSs of *DCS*, *CURS1*, *CURS2*, *CURS3*, *DCS1* (KM880191.1) and *DCS2* (AB535216.1) from *C. longa,* type III *PKS* **(**polyketide synthase) gene from *Musa accuminata* (GU724611.1), 4 newly obtained genes from *C. zedoaria* were subjected to phylogenetic analysis using MEGA7 software. A fungal *PKS* gene from *Alternaria alternate* (JX103640.1) was used as an outgroup to create a rooted tree. The phylogenetic tree was created using Marximum Likelihood method [24, 25] with bootstrap support of 2000 replicates.

2.5. Gene Expression

The expression level of curcuminoid genes in various samples was analysed by RT-PCR with the primers that were designed to amplify their specific regions (Table **2**). Primer design was carried out using Primer BLAST. The predicted mRNA sequences of *CzDCS*, *CzCURS1*, *CzCURS2*, and *CzCURS3* were used as templates. The primers were restricted to regions of high variation among these sequences to ensure that each PCR product is truly an indicator of its full-length sequence.

200 mg of callus or 100 mg of rhizome (10 month old) of *C. zedoaria* was ground to a fine powder by using liquid nitrogen. Total RNA of tissues was extracted by InviTrap Spin Plant RNA Mini Kit (STRATEC Biomedical, Germany) and 1st cDNA strand was synthesized by First Strand cDNA Synthesis Kit (Thermo Scientific), both under the manufacturer's instructions.

PCR amplification was performed as follows: 50 ng cDNA, 10 pmol each primer, 6 µL 2× GoTaq® Green Master Mix (Promega), and ddH_2O in a final volume of 12 µL. The reaction was performed in a thermocycler (Veriti 96 Well Thermal Cycler, ABI) with a template denaturation of 95°C for 2 min; followed by 30 cycles of 95°C for 30 s, 50-55°C

for 30 s (Table **2**), and 72ºC for 30 s; and a final extension of 72ºC for 10 min. Three microliters of each PCR product were used for electrophoresis on 1% (*w/v*) agarose gel at 70 V and intensities of DNA bands were calculated using ImageJ program (ver. k 1.45).

2.6. High Pressure Liquid Chromatography

Fresh samples (callus and rhizome) were dried at 50°C to a constant weight, and then ground into a fine powder. One gram of sample was immersed in 10 mL 70% ethanol and shaken at 120 rpm/37°C for 48 h. The extract was then filtered by Whatman paper (No. 1) and concentrated at 70°C using a vacuum rotary concentrator (Heidolph, Germany). The concentrate was dissolved in 70% ethanol to 5 mL (curcumin extract) and filtered through Minisart 0.25 µm membrane (Sartorius, Germany) for subjecting HPLC at ambient temperature by using a Vertisep GES C18 column (5 µm, 4.6×150 mm).

HPLC condition was as follows, flow rate: 1.2 mL/min, run time: 30 min, detector wavelength: 420 nm, stationary phase: silica gel (reverse phase) and mobile phase of acetonitrile:2% acetic acid (50:50, v/v). Twenty microliters of curcumin extract were injected in the chromatographic column using Hamilton syringe. HPLC analysis was performed on an LC-20A prominence system (Shimadzu, Japan). All solvents were of analytical grade and were purchased from Sigma and Merck & Co., Inc. Curcumin solution in ethanol from Sigma was used as a standard for the determination of curcumin in the extract.

3. RESULTS AND DISCUSSION

3.1. Isolation of Curcuminoid Genes

Genomic amplification of zedoary by PCR with primers that were designed based on the 5'- and 3'-untranslated regions of *DCS*, *CURS1*, *CURS2* and *CURS3* genes from *C. longa* exhibited specific DNA bands in electrophoretic image with the sizes as expected. The putative full lengths of *CzDCS*, *CzCURS1*, *CzCURS2* and *CzCURS3* genes were about 1.4 kb, 1.25 kb, 1.3 kb and 1.25 kb, respectively (Fig. **2**).

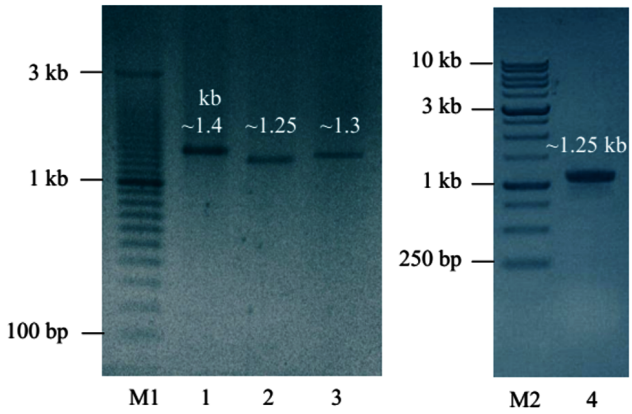

Fig. (2). PCR amplification of curcuminoid genes from *C. zedoaria*. M1 and M2: DNA size marker (100 bp BioRad and 1 kb DNA Ladder Geneaid, respectively), 1: *CzDCS*, 2: *CzCURS1*, 3: *CzCURS2*, 4: *CzCURS3*.

Nucleotide sequences of these genes show near identity (approx. 99%) with corresponding genes from *C. longa*. Our prior sequence analysis showed that all species within *Curcuma* genus share very high sequence identity even for non-coding sequence. For example, 5S ribosomal RNAs of *C. longa* (LT853920.1) and *C. zedoaria* (KU936068.1) showed 92% identity. The *CzDCS, CzCURS1, 2* and *3* genes were deposited in the database of GenBank with accession numbers of MF663785, MF402846, MF402847, and MF987835, respectively. Based on such high sequence identity among species of this genus, we assume that they also have high sequence identity for protein coding sequences. This forms the basis for our primer design strategy to clone genes involved in curcuminoid biosynthesis in *C. zedoaria*.

One problem with primer design for cloning each individual gene from *C. zedoaria* is that these genes display high sequence identity, particularly in the starting and stopping region of the CDS. Since there are no full-length mRNA sequences of these genes in GenBank from any species within the genus, we had to rely on the sequences of these genes from *C. longa*. However, entries for these genes on GenBank only contain CDS. To obtain flanking 5' and 3' UTR (untranslation region), we subjected *C. longa* gene sequences to blastn against EST database of *C. longa* and identify ESTs that highly likely belong to these genes. These ESTs were subsequently joined with their corresponding CDSs to create longer contigs. Since UTRs of these genes show strong variation, primers for complete CDS were designed in these regions to ensure specificity of PCR. Our PCR results showed that products were highly specific for all genes (Fig. **2**).

Studying a related species, *M. accuminata* showed that this species has 3 copies of *CURS3*-like gene (LOC103968790, LOC103968789 and LOC103968788), all located in chromosome 10, and 5 copies of *CURS2*-like gene (LOC103976634, LOC103976805, LOC103986053, LOC103987346, LOC103968787) on chromosome 2, 5, 6 and 10. However, to answer the question about the number of copies of the curcuminoid gene in *C. zedoaria* certainly, a thorough examination using genomic approach should be employed.

To identify intron/exon in our genomic DNA sequences of *CzDCS*, *CzCURS1*, *CzCURS2* and *CzCURS3*, we used several strategies to ensure high accuracy of identity assignment. Firstly, genomic sequences of these genes were aligned with CDS sequences from corresponding genes of *C. longa* and regions that do not align are assigned as 5' UTR and 3' UTR and introns. In addition, we also used blastx and FGENES to confirm the results obtained by the alignment method. By doing this, we have assured that our predictions are as accurate as possible. In addition, any significant errors in the sequencing results would affect the outcome of intron/exon prediction and could be traced and corrected in the sequencing results. The results showed that *CzCURS1*, *2* and *3* have one intron while *CzDCS* has two introns. This agrees with corresponding genes from *M. accuminata*. The theoretical boundaries of intron/exon for all four genes together with their protein domain arrangement are shown in (Fig. **3**).

Due to high sequence identity among *C. zedoaria* curcuminoid biosynthesis genes, we employed phylogenetic analysis as a tool to correctly assign identity for these genes.

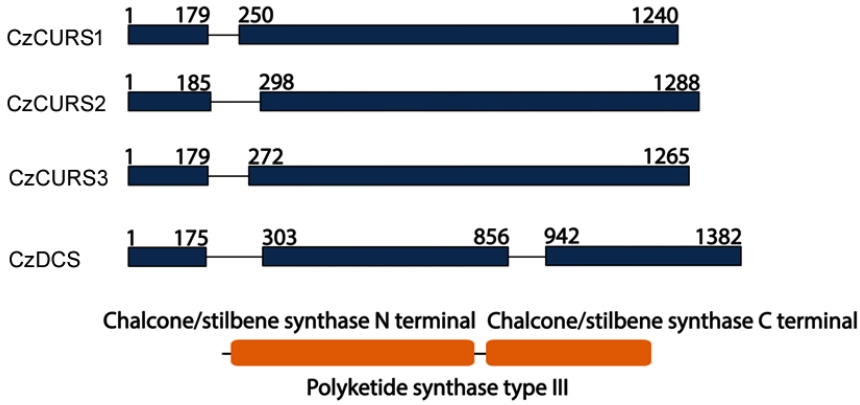

Fig. (3). Schematic diagram of intron/exon arrangement. The diagram shows intron (line), exon (box) and their boundaries in four curcuminoid biosynthesis genes in *C. zedoaria*. Number 1 indicates the first nucleotide of the start codon. The general structure of the encoding proteins is also shown below. Domain assignment was obtained using Interproscan (http://www.ebi.ac.uk/interpro/search/sequence-search).

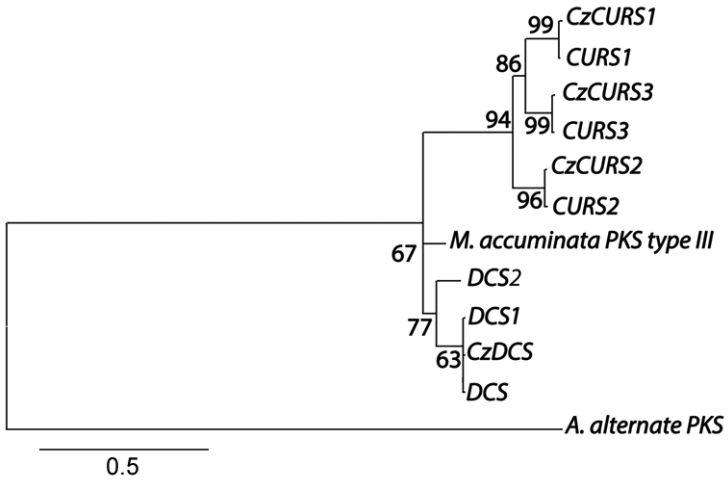

Fig. (4). Molecular phylogenetic analysis of curcuminoid genes. The evolutionary history of curcuminoid genes from *C. zedoaria* and *C. longa* was inferred by Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-6209.5470) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.9110)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, with a scale bar at the bottom to indicate the unit length of the branch. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1188 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*. 2015).

CDS sequences of *DCS*, *CURS1*, *CURS2* and *CURS3* from *C. longa* and *C. zedoaria*, together with a type III polyketide synthase gene from *M. accuminata* and a fungal polyketide synthase gene (outgroup) from *A. alternate* were subjected to analysis by MEGA7 using Maximum Likelihood method with bootstrap support (2000 replicates). The results are shown in Fig. (**4**). From this result, we can see that corresponding genes cluster with very high bootstrap values. The tree helps show orthologs and paralogs of these genes in *C. longa* and *C. zedoaria*.

3.2. Expression of Curcuminoid Genes

To assess the expression of curcuminoid biosynthesis genes in *C. zed*oaria, we chose the RT-PCR method. Since using primers for cloning full-length genomic DNA sequences did not give us consistent results in the RT-PCR, we redesigned primers for amplifying much shorter products. The primers were designed in regions that showed high dissimilarity among the genes (to ensure specificity) and also resulted in amplicons that could be easily distinguished by naked eyes.

The data of RT-PCR of four curcuminoid genes (*CzDCS*, *CzCURS1*, *2*, and *3*) in Fig. (**5**) indicated all the genes expressed both in the rhizome and callus. Except for the *CzCURS1* gene, other genes (*CzDCS*, *CzCURS2* and *3*) expressed in the rhizome are stronger than callus with the intensity of 1.4-1.9 fold (Table **3**). The results suggested that the curcuminoid metabolism of *C. zedoaria* also occurs in

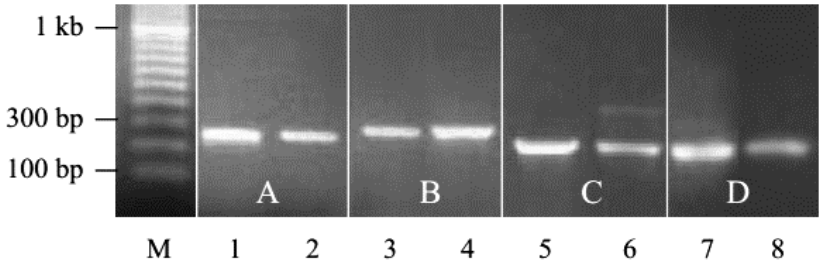

Fig. (5). Transcription expression of curcuminoid genes in various tissues of *C. zedoaria*. M: DNA size marker (1 kb), 1-3-5-7: rhizome, 2-4-6-8: callus, A: *CzDCS*, B: *CzCURS1*, C: *CzCURS2*, and D: *CzCURS3*.

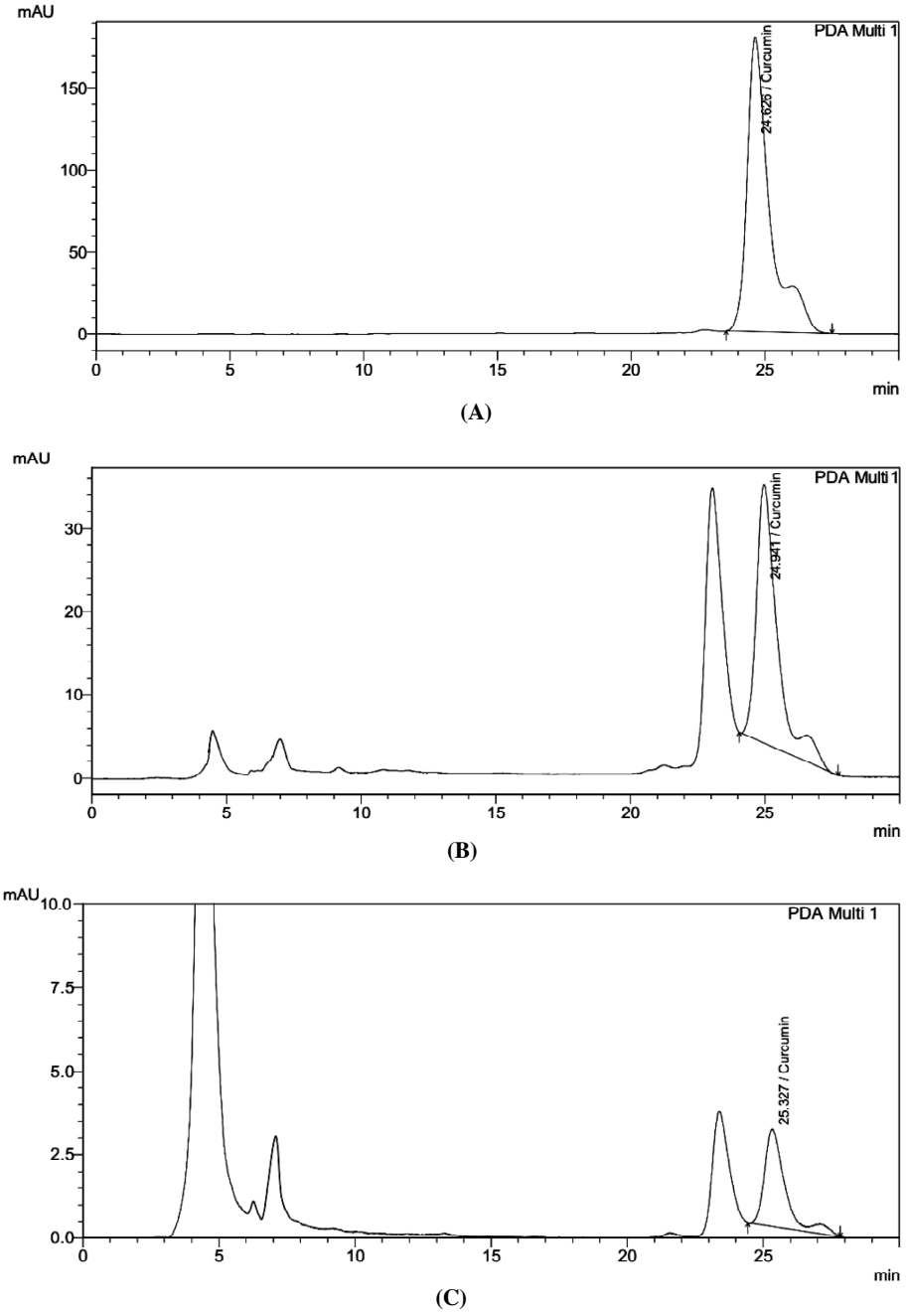

Fig. (6). (**A**) HPLC chromatogram of standard curcumin, (**B**) HPLC chromatogram of the extract from 10 month old *C. zedoaria* rhizome, and (**C**) HPLC chromatogram of the extract from 14 day old *C. zedoaria* callus.

Table 3. Intensities of DNA bands from semi-quantitative RT-PCR of the specific regions of curcuminoid-biosynthesis genes.

Gene	Sample	Lane	Intensity	Ratio of Rhizome to Callus
CzDCS	Rhizome	1	4061.3	1.8
	Callus	2	2231.9	
CzCURS1	Rhizome	3	2288.1	0.6
	Callus	4	3946.7	
CzCURS2	Rhizome	5	5018.3	1.9
	Callus	6	2641.9	
CzCURS3	Rhizome	7	4684.5	1.4
	Callus	8	3281.8	

in vitro culture, and callus is a suitable material source for establishing plant cell suspension culture to produce curcumin. The electrophoretic imaging shows the lengths of specific regions for *CzDCS* and *CzCURS1*, *2*, *3* being approximately 272 bp, 286 bp, 211 bp and 202 bp, respectively as expected. Katsuyama *et al.* [3, 7] confirmed the expression of *DCS*, *CURS1*, *CURS2* and *CURS3* genes in the rhizome and leaves of *C. longa* by qPCR (quantitative PCR). Behar *et al.* [4] also observed transcription of *DCS*, *CURS*, *CURS2* and *CURS3* genes in the rhizome and leaves of *C. caesia*, in which *CURS* transcription of the 5 months old rhizome was 4-fold higher than that of the 10 month old rhizome.

HPLC analysis indicated that both the rhizome and callus extracts contained curcumin peaks with similar retention times as the standard curcumin, and curcumin contents in the 10 month old rhizome were about 10 times higher than the 14 day old callus (0.78 mg/g vs 0.08 mg/g dry weight) (Fig. **6**). Curcumin is a major component of curcuminoid compounds and its presence in callus was further confirmed through RT-PCR analysis of the expression of curcuminoid genes (Fig. **5**). However, the area of the peaks in Fig. (**6B** and **6C**) suggests that biotransformation may occur during callus culture. Previous reports of Sakui *et al.* [26] and Loc *et al.* [22] also found biotransformations of sesquiterpenoids in cell suspension culture of zedoary that originated from callus. According to Muffler *et al.* [27], biotransformation was required to obtain bioactive compounds from precursor molecules in triterpene production by plant tissue engineering. Study of Dvořáková *et al.* [28] also showed the biotransformation of a monoterpene mixture in cell suspension cultures of two conifer species, *Picea abies* and *Taxus baccata*.

Prior to this study, we assumed that the curcuminoid biosynthesis pathways are similar among *Curcuma* species. Although this study is not sufficient to reach such a conclusion, the current evidence has corroborated our assumption. However, the exact sequences of genes involving in the pathway were hitherto unknown for *C. zedoaria*. This would hinder the analysis of transcriptional changes of relevant genes under different conditions using traditional approaches such as Northern blot, Real-time PCR or RT-PCR. Normally, the cloning of homologs from another species requires a complicated process of multiple sequence analysis and degenerate primer design. Based on the observation of the unusually high sequence identity (92%) between *C. longa* and *C. zedoaria* even in the noncoding DNA, we were able to simplify the cloning process of homologous genes in *C. zedoaria* by designing specific primer instead.

CONCLUSION

The sequence information of *DCS* and *CURS1-3* genes in *C. zedoaria* will be very valuable for a subsequent study on the effects of elicitors on the transcription of genes involved in curcuminoid biosynthesis pathway. It also allows cloning and expression of these genes in a new host to facilitate biochemical analysis of the encoded enzymes. Finally, it facilitates the molecular investigation of differential expression among different *Curcuma* species to elucidate the precise mechanism of gene regulation that controls the accumulation of curcumin in these species.

CONSENT FOR PUBLICATION

All authors have agreed to submit this manuscript to the Current Pharmaceutical Biotechnology.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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