Pertanika J. Trop. Agric. Sci. **31(1)**: 101 - 105 (2008) ISSN: 1511-3701

Cloning of a Near Complete *Isochorismate synthase* (ICS) cDNA from *Morinda citrifolia* L.

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ABSTRACT

Isochorismate synthase (ICS) is a key enzyme that catalyses the conversion of chorismate to isochorismate which is then channelled to other secondary product such as the anthraquinones. A near complete cDNA was isolated through RT-PCR technique. Characterization of this gene is important in characterising its role in the production of anthraquinones in the Rubiaceae plant family. Anthraquinones are known for their medicinal properties and can be found in Rubiaceae especially in roots. In this study, total RNA was extracted from roots of 'mengkudu' using modified CTAB method. The total RNA was subjected to first strand synthesis using oligo-dT_{18} primer and M-MuLV reverse transcriptase. Subsequently, PCR technique using primers designed from conserved ICS domains from other plants were used to isolate an internal conservative region of 426 bp. The cDNA was subsequently sequenced and verified using BLASTn program through the NCBI Genebank database, which showed a high sequence identity (72%) to the ICS from *Catharanthus roseus*. Based on this sequence, 3'RACE was performed to obtain the 3'-end of the gene and a 1036 bp 3'-fragment was generated. Apart from that, another PCR managed to generate a fragment of 491 bp upstream of the cDNA. Both fragments were sequenced and verified. Contig analyses and assembly of the partial cDNAs generated showed a near complete cDNA of 1872 bp. Sequence analysis of this partial cDNA showed a high degree of identity with ICS cDNA from other plants with the highest identity of 72% with ICS from *C. roseus*. Deduced amino acid showed a high similarity with *Rubia cardifolia* ICS of 85%.

Keywords: *Isochorismate synthase*, Rubiaceae, 'mengkudu', anthraquinones, PCR, 3'RACE

INTRODUCTION

'Mengkudu' or *Morinda citrifolia* is a plant belonging to the family of Rubiaceae. The plant has a height of between 3 and 12 m, the leaves are oval shaped and the fruit is fleshy, bumpy-structured, green in colour and changing to translucent upon ripening and produces odour. 'Mengkudu' is used in many cultures because of its medicinal properties for both traditional and modern medicinal purposes. Various parts of the plant are used such as the juice of the fruit and leaves (for arthritis, asthma, lumbago, regulate menstrual flow, heated leaves are used to relieve coughs, nausea, and colic); flowers, roots and bark (to treat eye problems, skin wounds, throat problems, respiratory ailments, constipation, stomach pains).

Modern research in 'mengkudu' is focussed in the application of the secondary products activity for various illnesses. Research on 'mengkudu' is mainly focussed on the therapeutic properties of its secondary products. 'Mengkudu' properties that have been reported include; analgesic and sedative properties (Younos *et al.*, 1990); anticancer properties (Hirazumi *et al.*, 1994); inhibitory properties against virus early-antigen activation (Hiramatsu *et al.*, 1993). Bioactive compounds from 'mengkudu' in the form of extracts have also been used to search for anti–malarial (Ancolio *et al.*, 2002; Tona *et al.*, 2001), anti-microbial (Jayasinghe *et al.*, 2002), anti-inflammatory (McKoy *et al.*, 2002) and anti-diabetic properties (Olajide *et al.*, 1999). In addition, 'mengkudu' has potential use in treating

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hyperkalemia (Mueller *et al.*, 2000) and cancer preventive treatment (Wang and Su, 2001) including promotion of immune responses in cancer treatments (Wong, 2004).

Metabolism and production of anthraquinones in 'mengkudu', which is normally stored in the root of the plants is of particular interest (Stalman *et al.*, 2003). In the plant family Rubiaceae that include species such as *Morinda*, *Rubia*, *Cinchona* and *Galium* species, anthraquinones are considered to be synthesised from chorismate (Han *et al.*, 2001). In this study, a near complete cDNA encoding *isochorismate synthase* was isolated and characterised. The enzyme is in the shikimate pathway that is involved in the biosynthesis of both primary and secondary metabolites. The ICS is involved in the conversion of chorismate to isochorismate and subsequently the conversion to 2,3-dihydroxybenzoic acid, phylloquinones and anthraquinones (Poulsen *et al.*, 1991).

Until now, there is no any information about gene encoding ICS from Morinda in gene bank but limited information about *ICS* in others plants is available. Recently some *ICS* cDNA have been isolated from plant species such as *Arabidopsis thaliana* (AF078080), *Catharanthus roseus* (AJ006065), *Capsicum annuum* (AY743431), and partial sequences from *Nicotiana tabacum* (AY740529) and *Rubia cardifolia* (EF090619). Isolation of *ICS* cDNA will enable a better understanding of the role(s) of the enzyme in production of anthraquinones in 'mengkudu'.

MATERIALS AND METHODS

Plant Materials

Root samples of 'mengkudu' were collected from the UNIMAS Plant House. All the samples were washed with 70% ethanol then rinsed with distilled water before RNA isolation.

RNA Isolation

Total RNA from 'mengkudu' root tissues was extracted using a modified CTAB method described by Zeng and Yang (2002). The quality and quantity of the extracted RNA were verified by agarose gel electrophoresis and spectrophotometry.

First Strand cDNA Synthesis

Total RNA was treated with DNaseI (Fermentas) prior to cDNA synthesis. Five microlitres of DNaseI-treated total RNA was mixed with 1 µl oligo-dT_{18} primer (100mM), distilled water was added to bring to a final volume of 13 µl and heated at 70°C for 5 min. The mixture was cooled on ice for 5 min, followed by addition of 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTPs mix and 1 µl of M-MuLV Reverse Transcriptase (Fermentas) (200 U/ µl). The reaction was conducted in a final volume of 20 µl. Reverse transcription was performed for 60 min at 42°C and the reaction was stopped by heating at 70°C for 10 min. The single stranded cDNA was stored at -20°C until further use.

Internal Conservative Fragment Cloning

PCR was carried out in a total volume of 25 µl containing 2.5 µl of 10X buffer, 2 µl of 25 mM $MgCl_2$, 2.5 µl of 2mM dNTP mix, 10 pmol forward primer, 10 pmol reverse primer, 1 U of *Taq* polymerase (Fermentas) and 0.5 µl of first strand cDNA product. The PCR amplification was performed using a Mastercycler Personal unit (Eppendorf) with an initial denaturation at 94°C for 3 min, 35 cycles of 30 s denaturation at 94°C, 45 s annealing 55°C and 1 min 45 s elongation at 72°C and a final elongation at 72°C of 7 min. PCR of cDNA internal sequence was conducted using primers ICSha-2F (5'-TGGTTCCTCAG GTTGAGTTTGAT-3') and ICSha-3R (5'-TCTGGAGTGTTTCCAATGAATGC-3'). All primers that were used in this work was designed from sequence identity between *A. thaliana* [AF078080] and *C. roseus* [AJ006065].

3'RACE-PCR

Based on the internal ICS sequence obtained, 3'RACE was performed according to Ambion RLM-RACE Kit. The gene specific primers used in the RACE were designed based on the internal sequence of ICS obtained from the Internal Conservative Fragment. A combination of primers; ICSha-1F:ICSha-2R, ICSha-1F:ICSha-3R and ICSha-5F:ICSha-5R, were used for the 3'RACE and the PCR was performed according to the manufacturer's instructions with some modifications. The list of primer sequences are presented in Table 1. First strand synthesis was carried out according to the protocol mentioned above but using 3'Adapter primer instead of oligo-dT.

TABLE 1
Listing of primer name and sequences used in the PCR and 3'RACE

Primer name	Sequence 5' – 3'
ICSha1-F	GCATTGGCCATGGAACGTCT
ICSha2-R	ATCAAACTCAACCTGAGGAACCA
ICSha5-F	ACAGAACGACGTTGTCAGTGTT
ICSha5-R	AGGCTTCGTCATGTTCTCTTGT

Cloning PCR Product

PCR products were cloned into the pGEM-T Vector (Promega). The positive clones were screened via PCR using gene specific forward and reverse primers. Clones corresponding to the expected size were selected for DNA sequencing. The clones were grown overnight and plasmid isolation was performed according to the modified methods from Birnboim and Doly (1979).

DNA Sequencing and Bioinformatics Analysis

DNA Sequencing service was obtained from First BASE Laboratories Sdn Bhd. All sequencing reactions were performed on double-stranded plasmid DNA by using BigDye^{Ò} Terminator v3.1 Sequencing Kit and analyzed on ABI PRISMÒ 377 Genetic Analyzer. BLASTn, ClustalW ver1.82 and ChromasPro programmes were used for sequence analysis, editing and alignment.

RESULTS AND DISCUSSION

For cloning of ICS cDNA, a 2-step RT-PCR strategy was used. The initial step taken was to isolate the internal sequence by PCR amplification using specific primers designed based on the conserved region of complete coding sequences of ICS gene from the gene bank (*A. thaliana* [AF078080], *C. roseus* [AJ006065]). Primers ICSha-2F and ICSha-3R were used to isolate the internal sequence (*Fig. 1*) and 426 bp cDNA fragments (fragment B) was obtained and sequenced. This sequence was verified against published sequences in NCBI GeneBank database using the BLASTn program. This cDNA fragment showed a 72% sequence identity to the *ICS* cDNA derived from *C. roseus* (accession no. AJ006065). Apart from that, another cDNA sequence of 490 bp (fragment A) was isolated using primers ICSha-1F and ICSha-2R. An 891 bp cDNA (fragment C) was amplified using primer ICSha-1F and primer ICSha-3R. The fragment C sequence confirmed the sequence generated by fragment A and B, whilst fragment D was PCR to confirm the sequences between the junction of fragment A and B.

From this initial internal conservative sequence (fragment B), ICS1 primer was designed and used as forward primer to isolate the 3' end. Subsequent 3'RACE-PCR managed to produce a fragment with 1036 bp that includes the poly(A) tail (Plate1, fragment E). Sequence analysis of this fragment showed high identity with *ICS* from *C. roseus* (72%), *C. annuum* (65%) and *A. thaliana* (65%).

Contig assembly analyses of fragments A, B and E, showed a near complete ICS cDNA of 1872 bp size. Sequence analysis of this partial cDNA showed a high degree of identity with *ICS* open reading frame from other plant species.

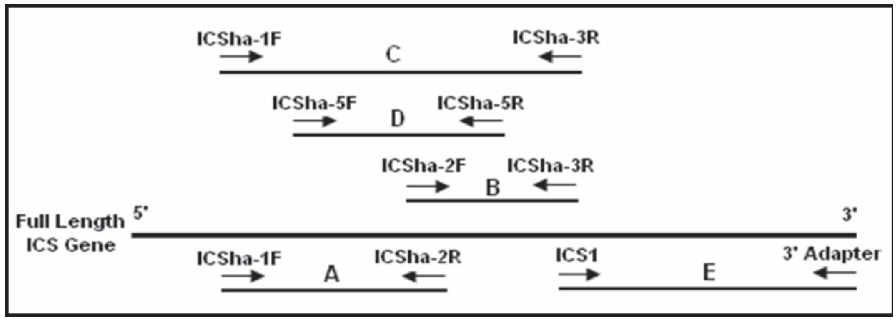

Fig. 1: Schematic representation of the cloning strategy of partial ICS cDNA by RT-PCR and 3'RACE. PCR was undertaken using different combinations of primer sets (Fragment A to D) and 3'RACE-PCR were performed to obtain the partial cDNA (Fragment E)

The highest identity was found to be 72% with ICS from *C. roseus*. Also ICS from *C. annuum* and *A. thaliana* were highly similar (67% and 62% respectively). A deduced amino acid sequence generated from the cDNA is also presented in *Fig. 2* and the percentage of amino acid similarity with other plant species is listed in Table 2 with the highest found to be with *R. cardifolia* (85%).

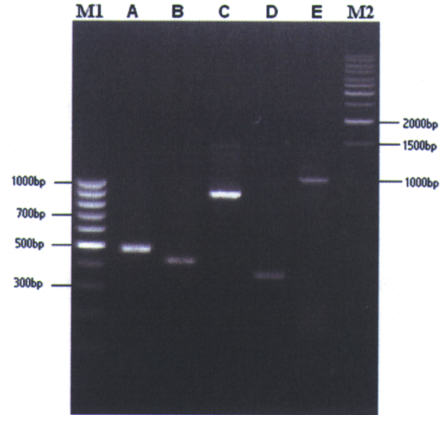

Plate 1: Amplification product of partial ICS cDNA via RT-PCR and 3'RACE. Lanes A to E represent the fragments labelled A to E in Fig. 1. M1 is the 100bp DNA ladder and M2 is 1kb DNA ladder (Fermentas)

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  1 ALA MER LSA AIA NMQ SDP SVF ESG IIR LEV PIE QQI KAL DWL QSQ DQS NVL PRC FFS GRK RIT ISD LSL NGL ING NGN GSS HVS TSI
 88 EQN DVV SVA GMG SAV LFR SLH PFS FDD WLS IRR FLS KNC PLI RAY GGI RFD GRA SIS PEW KSF GSF YFR VPQ VEF DEL EGS SKI AAT
175 IAW DNA LSC SYR SAI AAL KST MAK ITS VVT REH DEA SHM HIT RKA HVP SRT SWD VAV NRA LDR IKG WDS PLT KVV LAR SSQ VLT SRD
262 INP LTW LDT LKA DGN DVY QFC LQP PES PAF IGN TPE QLF RRD QSS IFS EAL AAT RAR GWS QSS DLQ IAH DLF SSP KDH HEF AIV REN
349 IRG KLQ AVC TSV AVK PEK VVR KLA RVQ HLY GRF SGR LHS EDD EFK ILS SLH PTP AVC GFP AED ARN FIT ETE MFD RGM YAG PVG FFG
436 GGQ SEF AVG IRS ALI GKD IGA LIY AGL GIV EGS DPS LEW EEL ELK ASQ FMK LMK LEV PAL ATI A** K*S GN* RFP EK* ITL GWY MRP
523 HLT DD* CHL QLK MLV RLR AHT RSK DFF FFL FFC FWV VFV *KK INL HIF GRP LDS ICF SFW GLI WVS SCK LSN SCK NPV TLS IYT YMV
610 RKK KKK KKK KKK KKK
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Fig. 2: Predicted amino acid sequence from partial ICS cDNA of M. citrifolia. *Asterix indicates possible termination codon for the deduced amino acid sequence*

TABLE 2
Sequence similarity of predicted amino acid from partial *ICS* cDNA of *M. citrifolia* with *ICS* from other plant species

Species	Length of deduced amino acid	% similarity	Accession no.
Catharanthus roseus	580	72	CAA06837
Capsicum annuum	576	66	AAW66457
Arabidopsis thaliana	622	59	NP_974143
Rubia cardifolia	252	85	ABK79678
Nicotiana tabacum	302	66	AAW67000

CONCLUSIONS

A near complete cDNA encoding *isochorismate synthase* have been isolated from *Morinda citrifolia*. The sequence shows a high sequence identity to the *ICS* cDNA from other higher plants (75% of nucleotide identity and 85% amino acid similarity to *C. roseus* and *R. cardifolia* respectively). A 5'RACE-PCR is currently in progress in order to get the full-length sequence of the gene.

ACKNOWLEDGEMENTS

This project was funded by a UNIMAS Fundamental Research Grant, 01(130)/520/2005(19).

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