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Molecular cloning of a cDNA fragment encoding 3-hydroxy-3-methylglutaryl CoA reductase in *kantkari* (*Solanum xanthocarpum*)

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Abstract

A cDNA fragment encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) was cloned from *kantkari* (*Solanum xanthocarpum*), a medicinal herb. cDNA was synthesized by reverse transcription polymerase chain reaction using specifically designed primers, with total RNA isolated from tender leaves. The 600bp amplicon obtained was cloned in pGEMT vector and sequenced, which revealed the presence of two open reading frames sharing homology with HMGR of other plant species. Sequence comparison of HMGR from eight different plant genera revealed that solanaceous plants belonged to a single cluster. Northern blotting followed by hybridization of total RNA using homologous probe confirmed the presence of corresponding mRNA. The full length gene could be cloned and utilized for imparting insect resistance to cultivated plants.

Keywords: Medicinal herbs, northern hybridization, reverse transcription polymerase chain reaction

Introduction

Solanum xanthocarpum Schrad. and Wendl., known as *kantkari*, is a spiny medicinal herb commonly found in India. Different parts of this plant are used in the treatment of diseases like fever, asthma, tuberculosis, kidney disorders, cough, constipation, tooth-ache, sore throat, rheumatism and gonorrhea (Govindan et al., 1996). The plant extract of *S. xanthocarpum* also possesses insecticidal and molluscicidal properties (Singh and Bansal, 2003). In addition, it is reported to show multiple resistances to major biotic stresses including insect pests like shoot and fruit borer (Sebastian, 2000). Phytoactive compounds extracted from this plant include solanocarpine, solanocarpidine, diosgenin, carpesterol and sitosterol and steroids (Josekutty, 1998). Previous workers (e.g., Sakai et al., 1981; Nelson et al., 1994) showed that isoprenoid biosynthesis of such defense proteins including phytoalexins is catalysed by the key enzyme, *3-hydroxy 3-methylglutaryl CoA reductase* (HMGR). In plants, however, this enzyme is encoded by a small gene family, the members of which are differentially expressed. Although genes encoding HMGR have been isolated and characterized from several plant species (e.g., Nelson et al. 1994; Jain et al., 2000; Kondo et al., 2003), no previous attempts were made to clone *hmgr* gene from *S. xanthocarpum*. In view of the potential medicinal and insecticidal importance of this species, an attempt has been made to isolate and characterize cDNA fragment encoding HMGR.

Materials and methods

RNA isolation

Total RNA was isolated by the single-step isolation method (Chomczynski and Sacchi, 1987) from the tender leaves of *S. xanthocarpum* treated with abscisic acid by dipping the roots in a 1000 ppm solution for 4h. Two hundred mg of tissue was ground in excess of liquid N_2 and RNA was isolated using TRIzol

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reagent (Invitrogen, USA). All glassware and reagents used in the process were treated with 0.1% DEPC and autoclaved. The RNA was separated by 0.8% agarose gel electrophoresis in tris-borate EDTA (TBE) buffer at 5 v/cm for 3 h. Approximate RNA yield was determined using a spectrophotometer (Genesys 5, Spectronic Instruments, USA) by measuring absorbance at 260 nm.

Primer designing and cDNA synthesis

The mRNA and amino acid sequences for *hmgr* genes from various plant species available in the databank of National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) were used to locate conserved boxes by multiple sequence alignment through CLUSTALW 1.8 and primers with the following sequence were designed.

hmgr 3 (forward) 5'
ACAGAAGGATGTTTAGTGGCTAG 3' (Tm 63°C)
*hmgr*6 (reverse) 5'
CTTGCCATCATTTACAGCCTC C 3' (Tm 61°C)

cDNA synthesis was carried out using 2µg total RNA in 50 µl reaction mix using single step reverse transcription polymerase chain reaction (RT-PCR) kit (Qiagen, Germany) in a thermal cycler (MJ Research, USA). First strand synthesis was carried out at 50°C for 30 min and second strand synthesis at an annealing temperature of 58°C for 1 min. At the end of the run, reaction mix was loaded on 0.8% agarose gel and the amplicon eluted using gel elution kit (Qiagen, Germany) in 25 µl of elution buffer.

Cloning and sequencing

Three µl of RT-PCR product eluted from gel was ligated with pGEMT vector using pGEM-T Easy Vector (Promega, USA) and transformed into competent cells of *Escherichia coli* DH5α prepared by $CaCl_2$ treatment. Recombinants were selected through blue-white screening on Luria agar. Presence of the insert in single white colonies was confirmed by PCR with the same primer combination and sequenced using T7 primer at the DNA Sequencing Facility, Delhi University South Campus, New Delhi.

Theoretical analysis of the nucleotide sequence and deduced amino acid sequence was carried out using various algorithms online, e.g., nucleotide sequence analysis included homology search by BLAST (http://www.ncbi.nlm.nih.gov/blast), restriction analysis (http://www.seqtool/sdsc.edu/), construction of phylogenetic tree (http://www.ebi.ac.uk/clustalw), amino acid sequence by 'GOR' secondary structure prediction (http://seqtool. sdsc.edu/), InterProscan domain search (http:www.ebi. ac.uk/InterProscan) to locate the functional domains, domain structure analysis by CATH structural database (www.biochem.ucl.ac.uk/bsm/cath) and Kyte & Doolittle hydropathy plot (http://occawlonline.pearsoned.com).

Northern blotting and hybridisation

RNA was electrophoresed on 1% denaturing agarose gel in sterile water, containing 10% of 10X MOPS (pH 7.0, 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA) and 17.5% formaldehyde. Sample was prepared with RNA 4 µl, 5X MOPS 3.12 µl, formaldehyde 3.12 µl and deionized formamide 8.88 µl. Sample was heated at 60°C for 7.5 min, cooled in ice for 5 min and 4 µl loading buffer was added (MBI Fermentas, USA) using 1X formamide as running buffer. After a pre-run for 10 minutes in 1X MOPS, samples were loaded and electrophoresed at 5 v cm^{-1} for 3 h.

The gel was washed and immersed in 100 ml of 10X SSC for 45 minutes with gentle shaking. The separated RNA was transferred on to nylon membrane with 20X SSC overnight, and immobilized on nylon membrane by baking at 80°C for 2 h. The RT-PCR product cloned in pGEMT was reamplified using *hmgr* 3 and 6 primers, ^{32}P labelled by random primer labeling using random primer labeling kit (Bangalore Genei, India). The nylon membrane was immersed in 30 ml pre-heated pre-hybridization solution (6X SSC, 2X Denhardt's reagent and 0.1% SDS) for 4 h at 68°C. Heat denatured probe was added to 30 ml of preheated hybridization solution (6X SSC, 2X Denhardt's reagent, 0.1% SDS and denatured salmon sperm DNA). Hybridization was allowed to proceed overnight at 68°C with gentle shaking. Nonspecifically bound activity was removed

by washing the membrane for 20 minutes at room temperature in 1X SSC and 0.1% SDS followed by three washes at 68°C in 0.2X SSC and 0.1% SDS. The membrane was exposed to X-ray film (Kodak) for 48 h at -70°C to develop autoradiograms.

Results and discussion

Total RNA from the leaves of *S. xanthocarpum* yielded five intact bands of RNA with no genomic DNA contamination (Figs. 1-3); and the approximate RNA yield was 3µg μl^{-1}. RT-PCR reaction with primer combination *hmgr*3 and *hmgr*6 yielded a single crisp band of 600 bp (Fig. 2), designated as '*sxhmg* 36'. Plasmids from true recombinants also yielded the same 600 bp amplicon in polymerase chain reaction.

The cDNA fragment cloned length of the insert was 533 bp and had 7 open reading frames. Homology search using translated BLAST (*blastx*) revealed that the longest ORF of 402b (134 amino acid residues) showed more than 90% identity with HMGR in *Lycopersicon esculentum, Nicotiana tabacum* and *Solanum tuberosum* (Table 1). The ORF on +2 strand was 162b (53 amino acid residues) long and contained stop codon. This ORF also shared homology with HMGR from *N. tabacum, L. esculentum* and *Capsicum annuum*. Generally, plant HMGRs show maximum identity in the C-terminal

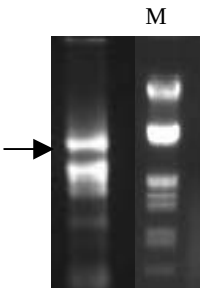

Fig. 1 Total RNA from *Solanum xanthocarpum*

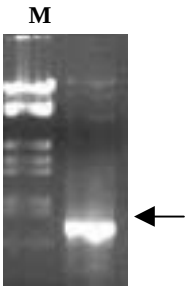

Fig. 2 RT PCR product with *hmgr* 3 and *hmgr* 6 primers

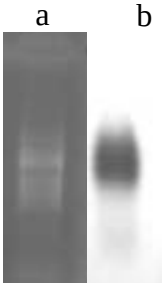

Fig. 3 Northern blot analysis
a: total RNA on denaturing gel b: autoradiogram

Table 1. Deduced amino acid sequence of ORFs and identity with other plant *hmgr* genes

ORF position and length in bp	Amino acid sequence	Details of sequences sharing homology			
		Accession No.	Identity (%)	Plant species	Function assigned
+3402	L R I P S T L R L F L L V F N K	AAB62581	93	*Lycopersicum esculentum*	
	S S R A R L Q S I Q C A I A G K	BAB20771	93	*Solanum tuberosum*	*hmgr*
	N L Y R D L A S T G D A M G M	AAQ11737	92	*Datura stramonium*	
	N M V S K G V Q N V L D Y L Q	AAD28179	90	*Capsicum annum*	
	N E Y P D M D V M G I S G N F	AAB87727	88	*Nicotiana tabacum*	
	C S D K K P A A V N W I E G R	AAT52222	85	*Catharanthus roseus*	
	G K S V V C E A I I K E E V V K	AAB69726	81	*Camptotheca acuminata*	
	K V L K T E V A A L V E L N M				
	L K N L T G S A M A				
+2162	M T R A P V V R F A T A K R A	AAL16927	87	*Lycopersicum esculentum*	*hmgr*
	A E M K F F V E D P I N F E T L	AAA93498	90	*Solanum tuberosum*	
	S S C V Q Q I K Q I C K I T E H	AAQ11737	63	*Datura stramonium*	
	S M C Y S W	AAD28179	65	*Capsicum annum*	
		AAL54879	61	*Nicotiana tabacum*	
		AAT52222	59	*Catharanthus roseus*	
		AAB69726	81	*Camptotheca acuminata*	

catalytic region and the membrane spanning region (Denbow et al., 1996).

Restriction analysis of the nucleotide sequence (GenBank Accession No: AAP15468) revealed that many of the commonly used restriction enzymes (*Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Sau*3AI and *Sma*I) did not have recognition sequences within. However, the enzyme *Alu*I had three sites, *Mbo*I two sites and *Bam*HI, *Dpn*I, *Hha*I, *Hinc*II and *Sac*I had one site each within the cloned sequence (Fig. 4). Eight plant genera sharing

Analysis of the amino acid composition showed that histidine residues were absent (Table 2). Generally among the members of HMGR family two histidine residues are conserved. One residue is transiently protonated during the conversion of HMG CoA to mevalonate (Liscum et al., 1985). Glycine that plays an important role in the maintenance of secondary structure constituted 6.21 mol% (10 residues). Cysteine residues, constituting 2.48 mol%, are important in the active site since HMGR is known to require high concentration of thiol-reducing agents (Liscum et al.,

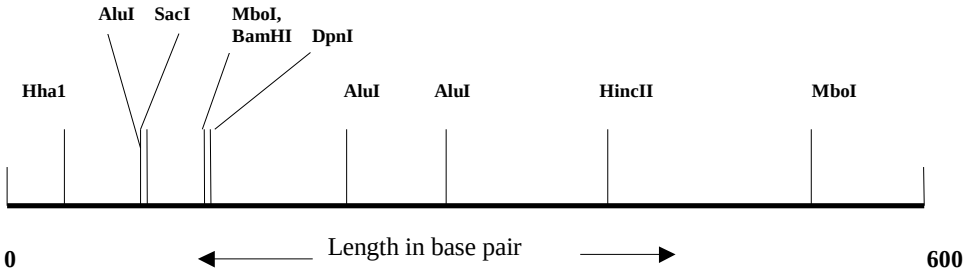

Fig. 4. Restriction map of *sxhmg 36*

homology with *S. xanthocarpum hmgr* gene showed that plants belonging to family Solanaceae, formed a single major cluster with two sub-clusters, implying genetic similarity among them (Fig. 5).

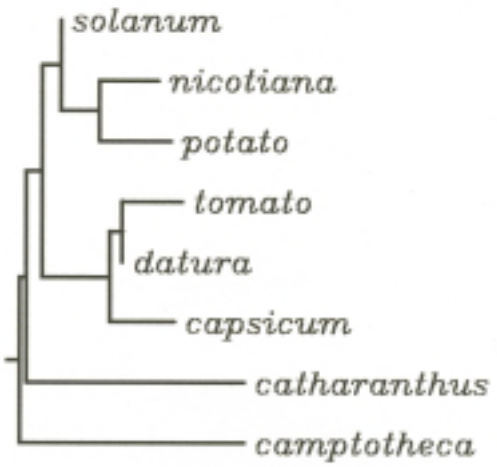

Fig. 5. Dendrogram of eight genera of plants sharing homology with *S. xanthocarpum* from sequence comparison of *hmgr* gene in BLAST

Table 2. Amino acid composition of HMGR protein in *Solanum xanthocarpum*

Amino acid	No. of residues	Mol (%)
Alanine	16	9.94
Arginine	8	4.97
Asparagine	10	6.21
Aspartic acid	7	4.35
Cysteine	4	2.48
Glutamic acid	10	6.21
Glutamine	4	2.48
Glycine	10	6.21
Histidine	0	0.00
Isoleucine	7	4.35
Leucine	13	8.07
Lysine	13	8.07
Methionine	10	6.21
Phenylalanine	8	4.97
Proline	4	2.48
Serine	11	6.83
Threonine	6	3.73
Tryptophan	1	0.62
Tyrosine	3	1.86
Valine	16	9.94

1985). Asparagine residues associated with glycosylation sites made up 6.21%.

Secondary structure analysis of the deduced amino acid sequence by GOR algorithm indicated that the protein consisted of 45% α-helix, 37% random coils and 18% β-sheets (Fig. 6). Motif scan of deduced amino acid sequence revealed presence of two motifs viz. HMGR signature 1 and HMGR family profile (Table 3). A comparison of the CATH structural database indicates oxido-reductase domain and domain 2 of HMGR chain A. These are similar to conserved regions in HMGR class I (containing N-terminal membrane region) and class II (soluble due to lack of membrane region), upon hybridization under stringent conditions with ^{32}P labelled homologous probe revealed positive signal in the autoradiogram (Fig. 3) confirming the presence of mRNA corresponding to HMGR protein.

In view of the multiple resistance of the plant to major biotic stresses, especially to insects, besides the medicinal properties of plant extracts, cloning *hmgr* gene from *S. xanthocarpum* and incorporating the same through genetic transformation would be useful in improving the biotic stress tolerance of cultivated plant species. In particular, attempts may be made for isolation of the full-length gene and cloning it under a strong constitutive or tissue-specific promoter, for better expression of the gene.

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Sequence   LLRDGMTRAP VVRFATAKRA AEMKFFVEDP INFETLSLVF NKSSRFARLQ
Structure  CCCCCCCCCH HHHHHHHHHH HHHHHHHCCC CCCCHHHHHH CCCCHHHHHH

Sequence   SIQCAIAGKN LYMRFSCSTG DAMGMNMVSK GVQNVLDYLQ NEYPDMDVMG
Structure  HHHHHHCCCC EEEEEEEEEE CCCEEEEEEC CCHHHHHHHC CCCCCCEEEE

Sequence   ISGNFCSDKK PAAVNWIEGR GKSVVCEAII KEEVVKKVLK TEVAALVELN
Structure  ECCCCCCCCC CCEEEEEECC CCCCHHHHHH HHHHHHHHHH HHHHHHHHHH

Sequence   MLKNLTGSAM A
Structure  HHHCCCCCEE C
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Fig. 6. Secondary structure of HMGR protein in *S. xanthocarpum* (H-α-helix, C-Random coils, E-β-Sheets)

predominant in eukaryotes and prokaryotes respectively (Goldstein and Brown, 1990)

No putative transmembrane regions could be detected in the amino acid sequence by the Kyte & Doolittle hydropathy plot. This could be because the N-terminal portion of the protein was not represented in the cloned sequence. In plant HMGRs two transmembrane regions are generally present anchoring the enzyme to endoplasmic reticulum (Basson et al., 1988). The denaturing gel stained with ethidium bromide confirmed the presence of RNA. Northern blotted nylon membrane

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References

Basson, M.E., Thorsness, M., Finer-Moore, J., Stroud, M.R. and Rine, J. 1988. Structural and functional conservation between yeast and human *3-hydroxy-3-methyl glutaryl*

Table 3. Details of motifs in deduced amino acid sequences of HMGR in *Solanum xanthocarpum*

Motif	Position	Description
HMG CoA Reductase 1	64-78	Hydroxy methyl glutaryl coenzyme A reductases signature 1
HMG CoA Reductase 4	1-161	Hydroxy methyl glutaryl coenzyme A reductases family profile

coenzyme A reductases, the rate limiting enzyme of sterol biosynthesis. Mol. Cell Biol., 8: 3797-3808.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156-159.

Denbow, C.J., Lang, S. and Cramer, C. 1996. The N-terminal domain of tomato 3-hydroxy-3-methylglutaryl CoA reductases. J. Biol. Chem., 271: 9710 – 9715.

Goldstein, J.L. and Brown, M.S. 1990. Regulation of the mevalonate pathway. Nature, 343: 425–430.

Govindan, S., Viswanathan, S., Vijayasekaran, V. and Alagappan, R. 1999. A pilot study on the clinical efficacy of *Solanum xanthocarpum* and *Solanum trilobatum* in bronchial asthma. J. Ethnopharmacol., 66:205-210.

Jain, A.K., Vincent, R.M. and Nessler, C.L. 2000. Molecular characterization of a 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) gene from mulberry (*Morus alba* L.). Plant Mol. Biol., 42:559-69.

Josekutty, P.C. 1998. Selection and characterization of variant *Solanum xanthocarpum* that overproduce steroids. Phyton Buenos Aires, 62: 125-130.

Kondo, K., Uritani, I. and Oba, K. 2003. Induction mechanism of 3-hydroxy 3-methyl glutaryl-CoA reductase in potato tuber and sweet potato root tissues. Biosci. Biotechnol. Biochem., 67: 1007-1017.

Liscum, L., Finer-moore, R J., Stroud, R. M., Luskey, K. L., Brown, M. S. and Goldstein, J. L. 1985. Domain structure of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a glycoprotein of the endoplasmic reticulum. J. Biol. Chem., 260: 522-530.

Nelson, A.J., Doerner, P.W., Zhu, Q. and Lamb, C.J. 1994. Isolation of a monocot 3-hydroxy-3-methyl glutaryl-coenzyme A reductase gene that is elicitor-inducible. Plant Mol. Biol., 25: 401-412.

Sakai, S., Tomiyam, K. and Doke, N. 1981. Effect of hyphal wall component of *Phytophthora infestans* on activity of rishitin synthetic pathway in a potato cell exposed directly to the component. Ann. Phytopathol. Soc. Japan, 47: 255-257.

Sebastian, S. 2000. Collection and characterization of land races of brinjal. MSc (Ag) thesis, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram. 121p.

Singh, K.V. and Bansal, S.K. 2003. Larvicidal properties of a perennial herb *Solanum xanthocarpum* against vectors of malaria and dengue/DHF. Curr. Sci., 84: 749-751.