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Isolation and characterization of lycopene β cyclase from *Capsicum frutescens*

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Abstract

Lycopene β cyclase (LCY-B) catalyzes the conversion of linear lycopene to cyclic β carotene, a crucial component of the photosynthetic machinery and a source of vitamin A for humans and animals. The gene was amplified in fragments using primers designed from conserved sequences of available nucleotide sequences from NCBI and a sequence of 1494 bp was observed upon amplification of the complete gene. The deduced amino acid sequence had a significant overall similarity with sequences of other *Capsicum* plants. It was observed that *C. frutescens* occurred in the same cluster as *C. annuum* and *C. baccatum,* while LCYB from all the other plants of the Solanaceae family were placed in one cluster signifying the conserved nature of the protein. The tertiary structure obtained from the protein was validated using the Ramachandran plot and ERRANT scores were determined. The protein function analysis gives insight into important parameters of the protein such as the number of cysteine residues, glycosylation sites, phosphorylation sites, *etc.*

Keywords: *Capsicum frutescens*, lycopene β cyclase, mRNA, amino acid, phylogenetics.

Introduction

Plants contain various phytoconstituents that have proved beneficial for human health and most of the drugs manufactured today are primarily or indirectly developed from plant sources. One such important group of phytoconstituents is the carotenoids. Carotenoids are lipophilic C40 isoprenoid polyene molecules that perform functions like attracting pollinators for plants, protecting plants against excessive light, and helps in attracting mates in certain animals (Vishnevetsky & Ovadis, 1999). There are more than 600 carotenoid pigments characterized up till now but only around 40 were found to be present in the human diet out of which only 10 were found to be

absorbed in human plasma and tissues (Chen, 2015). These molecules are important for the health of humans because they perform various health-promoting activities such as improving the immune system, acting as antioxidants, and helping minimize eye macular deterioration which results in the occurrence of impaired vision in aged individuals and also in the treatment of cancer and cardiovascular diseases (Nisar *et al.* 2015; Howard *et al.* 2000). Carotenoids are also vital economically with a worldwide projected demand of about 935 million US dollars in 2005 as a coloring agent and also as a source of nutritional supplements (Fraser & Bramley, 2004). Humans cannot produce carotenoids *de-novo* although carotenoids are significant in maintaining the health of humans, we rely on our diet to obtain these components (Fraser & Bramley 2004). For the normal functioning of the immune system, visual sense, and expression of genes the recommended dietary allowance (RDA) of carotenoids is specified in connection with retinol where 900 μg day^{-1} of provitamin A is recommended for males and 700 μg day^{-1} for females (Ellison, 2015). Most popular examples of carotenoid molecules include lycopene from tomatoes, capsanthin and capsorubin from *Capsicum*, β-carotene from carrots, and also the peculiar pink coloration of flamingos (Ngamwonglumlert & Devahastin 2018). Various studies have documented significant variation in carotenoid accumulation not only among different species of crops but also within the same species; few examples of this include carrots, potatoes, and peppers (Sommerburg *et al.* 1998; Lachman *et al.* 2016; (Nicolle *et al.* 2004; Guzman *et al.* 2010).

Peppers fall under the non-leafy vegetable category belong to *Capsicum* genus and act as a rich source of carotenoids. The *Capsicum* genus contains several species which are utilized all over the world for their flavoring property. Beyond their culinary use, these species are also valued as spices and for their medicinal properties. It has around 20 species out of which five most domesticated spices include *Capsicum annuum*, *Capsicum baccatum*, *Capsicum chinense*, *Capsicum frutescens*, and *Capsicum pubescens*. The *Capsicum* plant is established to be vastly valuable to humans due to its high antioxidant content owing to the presence of phytoconstituents such as flavonoids, carotenoids, and vitamin C (Berke & Shieh, 2012). Pepper fruits occur in different colors that range from ivory, yellow, or green to different variations of red, orange, and brown varying according to the stage of maturation (Nicolle *et al*. 2004; Tian *et al.* 2014).

The concentration of carotenoids is mostly based on the different gene expressions engaged in carotenoid biosynthesis (Hao & Chen, 2020). The increasing use of carotenoids in the food and pharmaceutical industry has opened new biotechnological approaches on the molecular level which facilitates different ways to increase carotenoid concentration in different plants (Saini*et al.*, 2015). One such approach is manipulating the genetic expression of carotenoid biosynthesis genes (Sandmann *et al.*, 2006). Much research has been carried out in this regard on *Capsicum annuum* but equal importance has not been given to *Capsicum frutescens*. With this information, the study was carried out to isolate the full-length gene

of LCYB and understand the regulation of carotenoid biosynthesis in *Capsicum frutescens.* Lycopene β-cyclase is a vital enzyme that is involved in the carotenoid process. LCYB is further responsible for the synthesis of crucial carotenoids such as α- and β-carotenes, lutein, and the important and unique capsanthin and capsorubin found in *Capsicum.*

Materials and methods

Plant material

The seeds were obtained from Thiruvananthapuram, Kerala, India (8° 31′ 26.9004′′ N 76° 56′ 11.8968″ E) and were grown in pots at The Institute of Science, Mumbai.

DNA isolation

Leaves of four weeks old plants were used for the genomic DNA extraction process, and the cetyltrimethylammonium bromide (CTAB) method was used with minor modifications. Liquid nitrogen was used to crush 10 mg of leaves in a mortar, and the powdered leaves were subsequently combined with an isolation buffer CTAB. Following that, 0.8% agarose gel was used to evaluate the quality of the isolated DNA. Then, using a Thermo Fischer Nano Drop Spectrophotometer, the isolated DNA was quantified.

Design of degenerate primers

As the sequence information on LCYB gene from the plant is not available in the public domain, the full length of the gene was amplified in fragments using primers designed based on homology with the conserved regions of available LCYB genes from *Capsicum* sp. and other solanaceous crops.

For designing primers, the available nucleotide sequences of lycopene β cyclase belonging to *Capsicum* genus and Solanaceae family were retrieved from NCBI https://www.ncbi.nlm.nih.gov/) and were aligned using MultAlin online tool http://multalin.toulouse.inra.fr/multalin/) the nucleotide sequences having conserved regions of these sequences were selected for designing of the primers. Once the gene was amplified in fragments and sent for sequencing to obtain the full-length sequence of LCYB in *C. frutescens,* a new set of primers was generated using conserved regions of the sequence obtained after aligning the gene fragments with other *Capsicum* species.

RNA extraction

Total RNA was extracted using young leaves of *C. frutescens* using TRIzol (Invitrogen) reagent using the manufacturers protocol.

cDNA synthesis

synthesis of cDNA using the extracted RNA was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was carried out by utilizing RevertAid M-MuLV RT enzyme with 100 μg of total RNA as template in a 20 μl reaction setup. 5 μl of the template RNA were used for the cDNA synthesis, along with 1 μl of Oligo dt primer, 1 μl of RiboLock RNase Inhibitor (20 U/μL), and 1 μl of RevertAid M-MuLV RT (200 U/μL). The reaction mixture was incubated for 60 minutes at 42°C.

PCR conditions

PCR amplification using degenerate primers was performed with a 25μL reaction mixture consisting of 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1X Taq. DNA polymerase buffer and 0.5 μM primers (Euroffins, India), 1.0 U Taq. Polymerase enzyme (Thermofischer) and 100-150 ng genomic DNA as template. The primers used for different fragments are mentioned in Table 1 and that for designing the complete length of the gene is given in Table 2. The PCR conditions for each fragment are mentioned in Table 3.

Table 1. List of primers used for different fragments of LCYB.

Lycopene beta cyclase (LCYB)	Forward and reverse primers (5′→3′)
lcybF1	ACGCTCTTGAGAACCCCAAA
lcybR1	GGTTTTCATGGATTGGCGCGAC
lcybF2	GTTGTTGTGGATCTTGCTGTGG
lcybR2	CACCACTGTCGCCTGAATA
lcybF3	GGTGGCTCGTTTAAGTCACTTGGG
lcybR3	CCTGGATGACAAGAAGCCA

Table 2. Primers used for full-length amplification of gene

Gene		Forward and reverse primers (5′→3′)
Lycopene β cyclase	Forward	ACGCTCTTGAGAACCCCAA
	Reverse	CCTGGATGACAAGAAGCC

Table 3. PCR conditions for amplification of fragments of LCYB

Step	Temperature (℃) for fragment 1	Temperature (℃) for fragment 2	Temperature (℃) for fragment 3	Time (min)	Cycles
Initial denaturation	95	95	95	5	
Denaturation	94	94	94	1	35 cycles
Annealing	54.2	58	60	1	
Extension	72	72	72	1	
Final extension	72	72	72	7	
Final hold	4	4	4	∞	

Table 4. PCR conditions for amplification of Full-length gene of LCYB

Step	Temperature (℃) for lycopene β cyclase	Time (min)	Cycles
Initial denaturation	95	5	35 cycles
Denaturation	94	1	
Annealing	60.0	1	
Extension	72	1	
Final extension	72	7	
Final hold	4	∞	

DNA Sequencing and sequence analysis

The amplified PCR product was analyzed on 1.5% agarose gel stained with 0.1μg/ml ethidium bromide using 1X TAE buffer. The obtained PCR product was sent for sequencing at Euroffins, India and the sequencing was performed using the Sanger sequencing method. The nucleotide sequence obtained was aligned with available NCBI sequences using multiple sequence alignment with hierarchical clustering and edited using Finch TV.

Phylogenetic analysis

The nucleotide sequences of lycopene β cyclase gene from various *Capsicum* genus were obtained from NCBI GenBank. The sequences were then used to perform multiple sequence alignment using the online tool MultAlin. The sequences were then used to perform phylogenetic analysis using MEGA XI software (Kumar *et al.*, 2018). The phylogenetic tree included nucleotide sequences from plants of Solanaceae family and an outgroup was also selected. The phylogenetic tree was constructed using Maximum Likelihood method and Kimura 2-parameter model with 1000 bootstrap.

Analysis of lycopene β cyclase primary structure

ExPASy ProtParam (Gasteiger *et al.*, 2005), was used to determine the primary structural analysis of lycopene β cyclase protein. The biochemical and biophysical characteristics such as molecular weight (MW), isoelectric point (pI), index of instability II-protein stability (Kunchur Guruprasad, & Bhasker Reddy, 1990), extinction coefficient (Gill & von Hippel, 1989), aliphatic index the relative protein amount covered by aliphatic side chains (Ikai, 1980) and the grand average of hydropathicity GRAVY (Kyte & Doolittle, 1982), while the half-life, and the number of positive and negative residues for the respective proteins were also determined.

Analysis of secondary structure

A secondary structure of the protein was predicted using Psipred http://bioinf.cs.ucl.ac.uk/psipred/) Mcguffin

et al., 2000) tool and self-optimized prediction method with alignment (SOPMA Geourjon & Deleage, 1995) to reveal its secondary characters like presence of α-helix and β-sheet for the protein amino acid series.

Tertiary structure prediction

The tertiary model of the deduced amino acid sequences was developed using the Swiss model software https://swissmodel.expasy.org/ (Arnold *et al.*, 2006). The structure obtained was further validated using Ramachandran's plot through the online tool (PROCHECK Laskowski *et al.*, 1996).

Protein functional analysis

The SS-linking of cysteine residues were predicted in the retrieved protein sequences using the CYC_REC function http://sunI.softberry.com/berry.phtml?topic. The prediction of glycosylation sites was performed using a NetNGlyc server http://www.cbs.dtu.dk/services/NetNGlyc/). NetPhos2.0 was used to predict possible protein phosphorylation sites (Blom *et al.*, 1999). The subcellular position of the protein was identified using ProtComp 9.0. Different motifs present in protein were evaluated using Psite (Solovyev VV, 1994) online tool. The InterproScan online tool was used to understand the function of the protein.

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Results and discussion

Amplification of full-length sequence of lycopene β cyclase

Different pairs of degenerate primers were designed to isolate the partial sequence of the gene in fragments depending upon the conserved regions of the gene. A total of 3 fragments were isolated using the primers with varying length as mentioned in Table 5, the gel images of the obtained fragments are given in Fig. 1 and the amplicon length for each fragment is given in Table 5. The gene fragments were later aligned with the already available sequences from NCBI using the MultAlin online tool. Using the conserved regions of the aligned sequences a new set of primers was designed to isolate the full-length of the gene. A sequence of 1494 bp was observed when run on 1.5 % Agarose gel electrophoresis (Fig. 2). The sequence obtained after sequencing was submitted in NCBI (Accession Number: OR044888.1). Using the ExPASy tool the nucleotide sequence obtained was translated into amino acid sequence which gave a sequence of 498 amino acids. The amino acid sequence obtained was compared with the protein sequence available for *Capsicum annuum* cultivars in NCBI using the MultAlin online tool and the results showed complete similarity with the deduced amino acid sequence of *Capsicum frutescens* for LCYB.

Table 5. Amplified fragment length of LCYB

Lycopene beta cyclase (LCYB)	Amplicon length (bp)	NCBI Accession Number
lcybF1	628	MT580127.1
lcybR1		
lcybF2	1013	OR044768.1
lcybR2		
lcybF3	682	OR044769.1
lcybR3		

Amino acid (aa) sequence and phylogenetic analysis

The deduced amino acid sequence was aligned with LCYB sequences of different *Capsicum* cultivars using MultAlin online tool. The Multiple sequence alignment (MSA) showed the conserved nature of LCYB where no indels were observed in the sequence (Fig. 3 A). The protein sequences were also analyzed for their conserved domains using NCBI- conserved domain database (CDD) search where LCYB showed PLN02463 (lycopene beta cyclase) as specific hits and NADB_Rossmann Superfamily confirming the protein under study to be involved in carotenoid biosynthesis (Fig. 3 B). The amino acid sequence was then used to perform phylogenetic analysis using MEGA XI software, for which the sequences were first aligned using MUSCLE and then used to build a phylogenetic tree with Maximum Likelihood method and Kimura 2-parameter model with 1000 bootstrap. The result showed *C. frutescens* in the same cluster as *C. annuum* and *C. baccatum,* while LCYB from all the other plants of the Solanaceae family were placed in one cluster signifying the conserved nature of the protein (Fig. 4.)

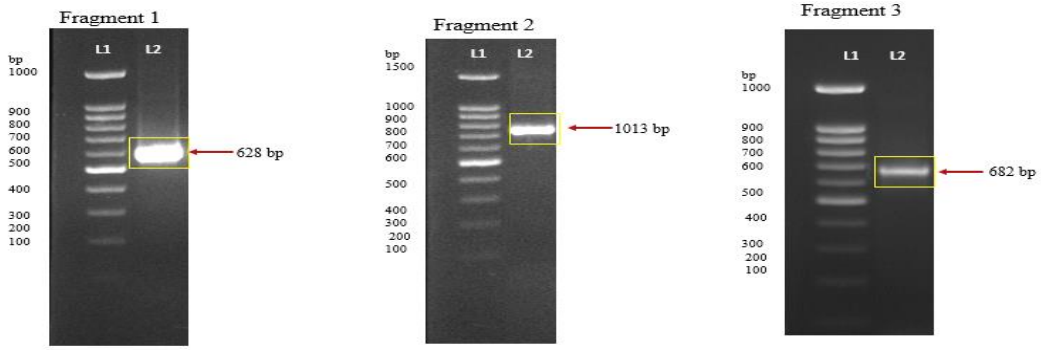


Fig. 1. Agarose gel electrophoretic analysis of PCR amplicon of lycopene β cyclase gene fragments amplified with primer**.** Lane 1 shows a standard 1500 bp ladder while lane 2 shows a PCR-amplified product of 628,1013 and 682 bp. After amplification samples were analyzed on 1.5 % agarose gel stained with 0.1 µg/mL of ethidium bromide.

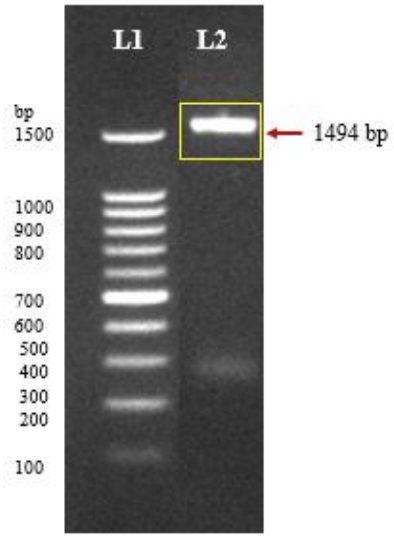


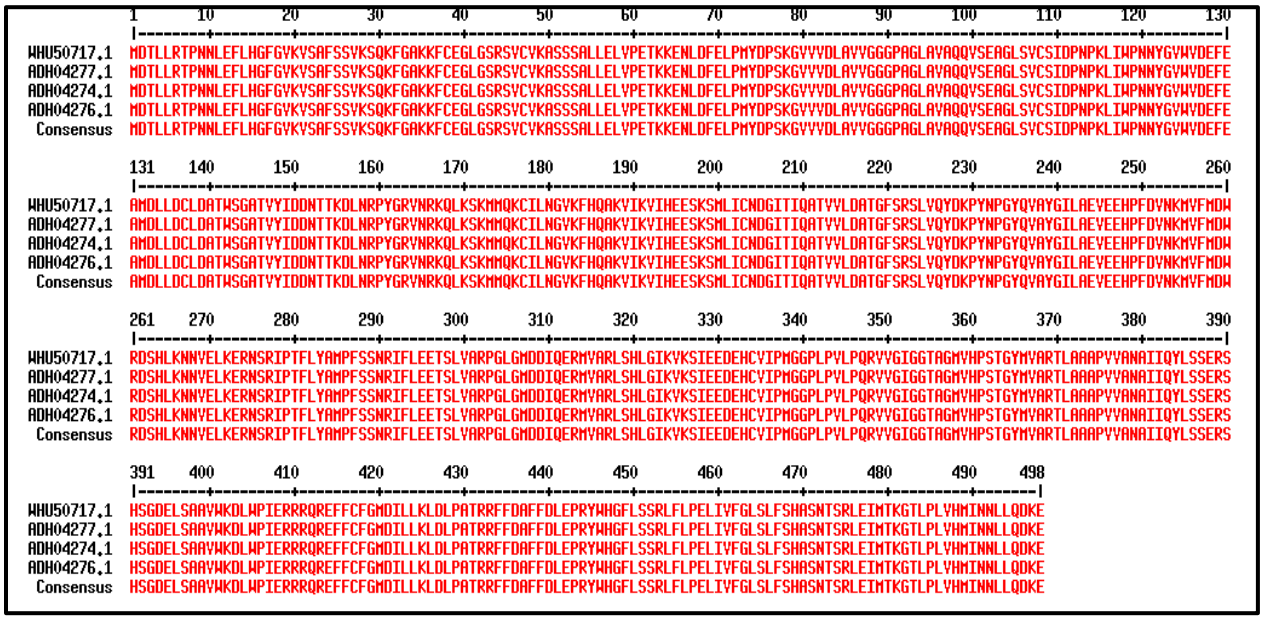
Fig. 2. Agarose gel electrophoretic analysis of full-length lycopene β cyclase gene PCR amplicon amplified with primer. *Lane 1 shows a standard 1500 bp ladder while lane 2 shows a PCR-amplified product of 1494 bp. After amplification samples were analyzed on 1.5 % agarose gel stained with 0.1 µg/mL of ethidium bromide.*

Lycopene β cyclase primary structure analysis

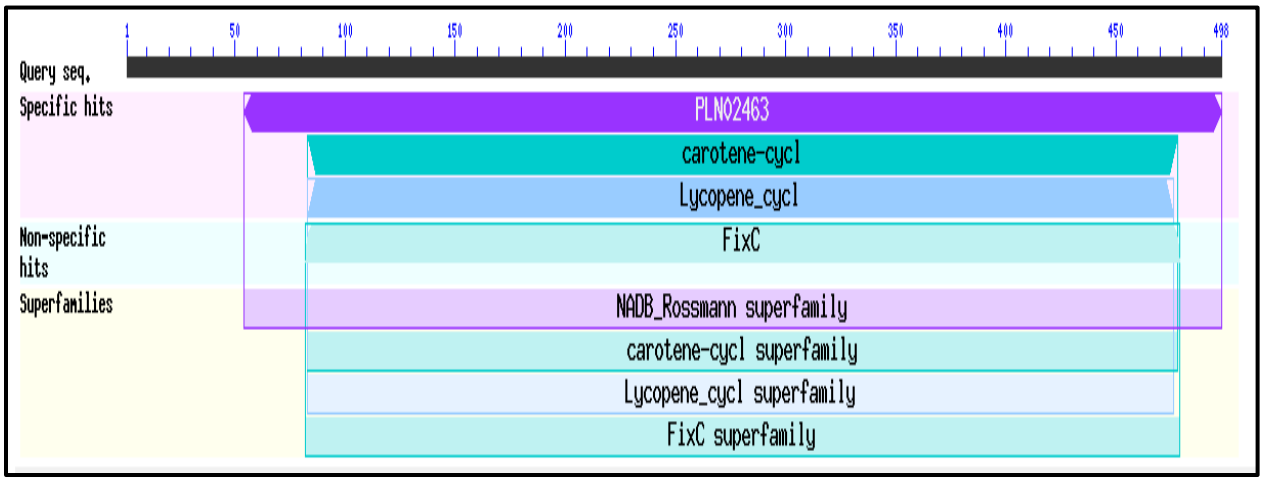
Amino acid composition

ExPASy ProtPram server was used to determine the amino acid alignment and physiochemical properties of *Capsicum frutescens* Lycopene β cyclase protein. The amino acid composition and physiochemical properties are shown in the Table (Fig. 5 and Table 6).

The isoelectric point which is described as the point where both the positive and negative charges are present in an equal amount is found to be 6.34 for LCYB protein which implies the amino acid sequences are acidic in nature. The computed instability index was found to be 37.73 which categorizes both the proteins as unstable which is similar to results observed in other plants (Zhao *et al.*, 2011). Another important parameter is the aliphatic index which indicates protein stability. The aliphatic index is described as the relative protein volume captured by the A (alanine), V (valine), L (leucine), and I isoleucine) aliphatic side chains in the protein (Kaur *et al.*, 2020). The aliphatic index was noted to be 92.55 along with the extinction coefficient of 75080 suggesting that the protein has stability for a wide range of temperatures. The grand average of hydropathicity (GRAVY) was observed to be -0.073 which further indicates protein solubility. The negative value for GRAVY analysis suggests that the protein is hydrophilic in nature and will have better performance while interacting in water (Wilkins *et al.*, 1998).



A



B

Fig. 3. A) MultAlin analysis of lycopene β cyclase protein sequence: *Capsicum frutescens* (WHU50717.1), *Capsicum annuum* cultivars (ADH04277.1 ADH04276.1, ADH04274.1

B) CDD search of *Capsicum frutescens* lycopene β cyclase protein (WHU50717.1) on NCBI.

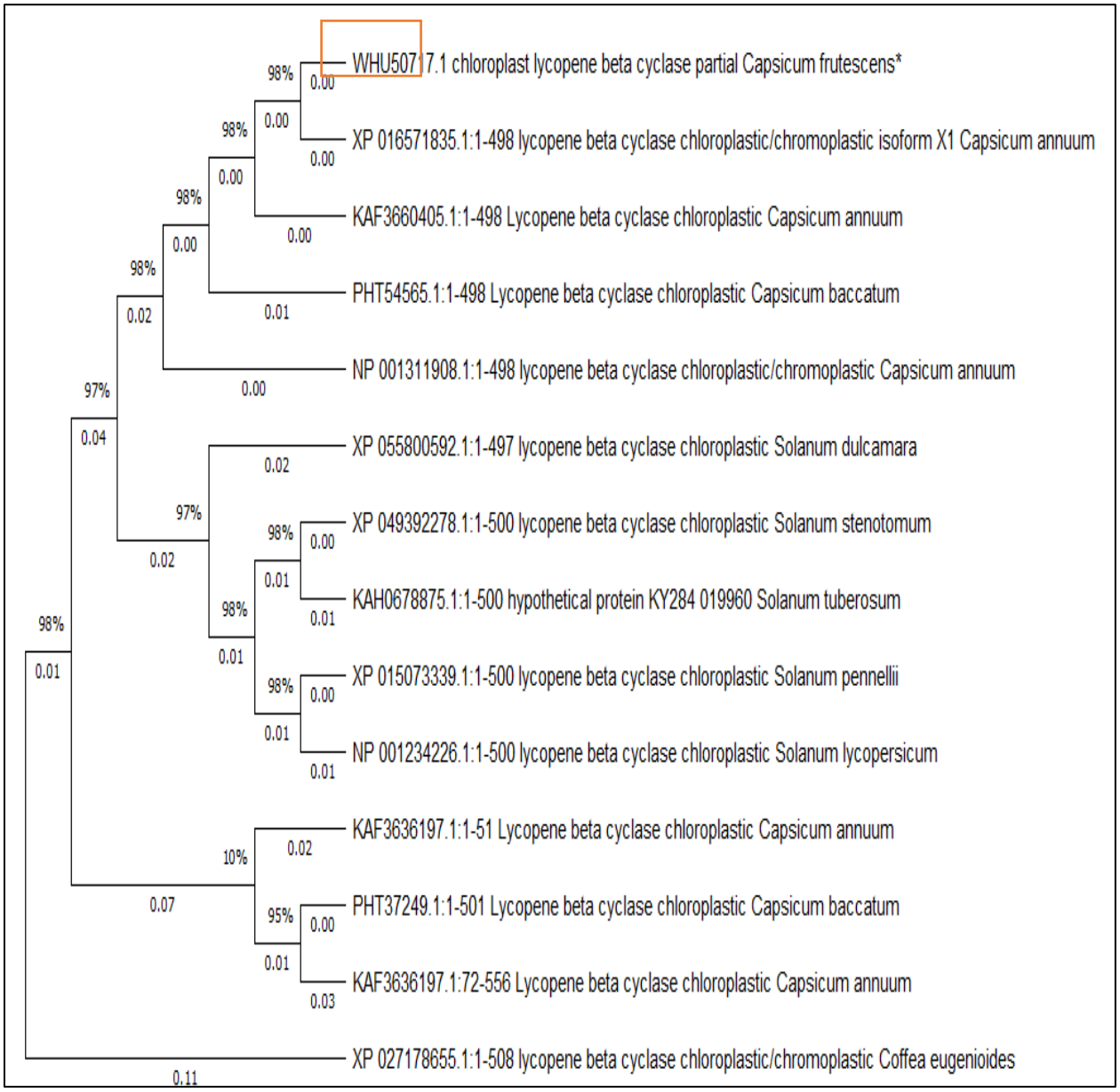


Fig. 4. Phylogenetic relationship of lycopene β cyclase protein among *Capsicum* species, plants belonging to Solanaceae family and outgroup taxa. The evolutionary history of lycopene β cyclase was inferred by using the Maximum Likelihood method and JTT matrix-based model [1]. The tree with the highest log likelihood -2704.24 is shown.

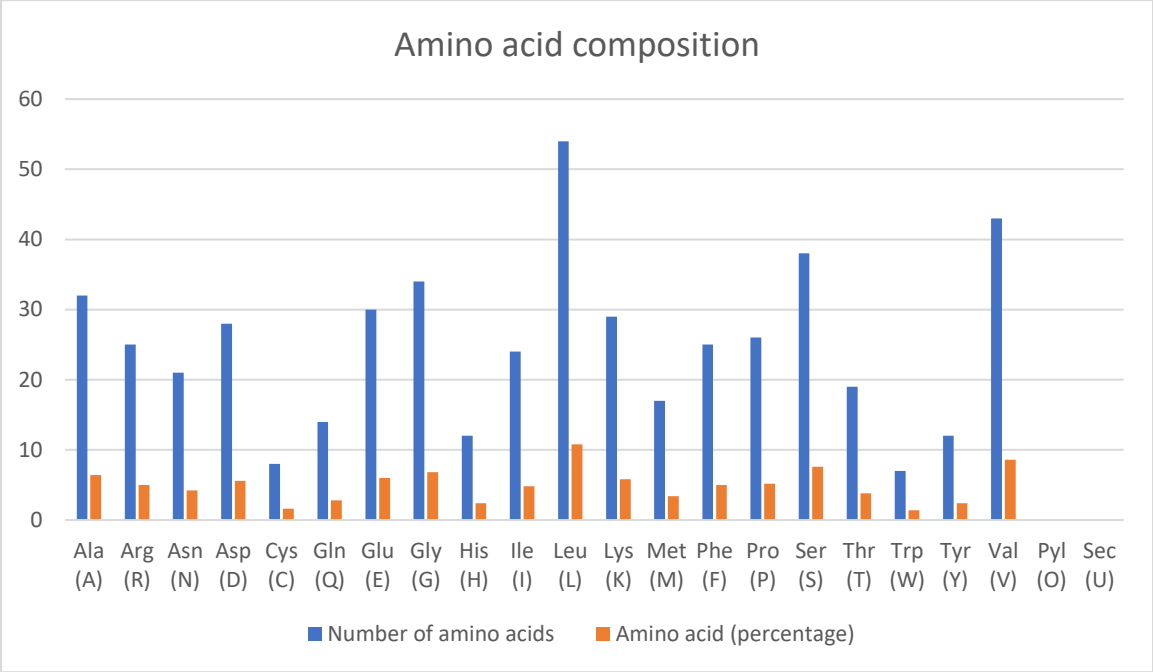


Fig. 5. Amino acid composition of LCYB

Lycopene β cyclase secondary structure analysis

The secondary structure of Lycopene β cyclase was predicted using the Protein Structure Prediction Server (PSIPRED v3.0) (Fig. 6.) where the structure showed presence of 15 α-helices, 22 strands, and 36 coiled structures. The secondary structure of lycopene β cyclase was also predicted using SOPMA online tool (Fig. 7.), where 39.56% sequences showed Alpha helix structure whereas 37.55% sequences showed random coiling. It also showed the presence of 16.87% sequences to be in the form of extended strands and 6.02% to be in Beta turn structures.

Tertiary structure

The tertiary model of the sequenced amino acids for lycopene β cyclase protein was developed using the Swiss model software (https://swissmodel.expasy.org/) (Fig. 8) and validated using the Ramachandran's plot through the online tool (http://mordred.bioc.ac,~rapper/rampage.php). In SWISS-MODEL the structure was generated through homology modelling using Q43415.1.A Lycopene beta cyclase, chloroplastic/chromoplastic AlphaFold DB model of LCYB_CAPAN gene: LCY1, organism: *Capsicum annuum* (*Capsicum* pepper) as a template to generate the protein structure. The template showed 100% sequence identity with the deduced protein sequence of LCYB of our study. The model was further verified using ERRANT which gave a score of 97.0655 further supporting the model obtained. The PROCHECK analysis of the protein sequence gave a Ramachandran plot (Fig. 9) with 88.1% residues present in the favored regions emphasizing the quality of the model obtaine.

Table 6. Lycopene β cyclase physiochemical properties

Properties	LCYB
Number of amino acids	498
Molecular weight	55626.23
Theoretical pI	6.34
Negatively charged residues (Asp + Glu)	58
Number of positively charged residues (Arg + Lys)	54
Formula	$C_{2498}H_{3930}N_{668}O_{719}S_{25}$
Ext. coefficient	75080
Abs 0.1%	1.548
Estimated half-life mammalian reticulocytes, (*in vitro*)	30 hours
Instability index	37.73
Aliphatic index	92.55
GRAVY	-0.073

Protein function analysis

LCYB showed 8 cysteine residues at positions 38, 47, 108, 137, 177, 203, 334, and 417. The next parameter considered during functional analysis was glycosylation which is one of the important post-translational modifications that is necessary for the folding of a protein, to determine its half-life, and for its transport. Lycopene β cyclase showed glycosylation at position 152 and 472, but the glycosylation potential was observed to be below the threshold value at position 472. Next important post-translational modification of plant protein considered for functional analysis was phosphorylation. The NetPhos2.0 results showed all LCYB possessed serine, threonine, and tyrosine as possible phosphorylation sites. The location of the protein was found to be extracellular secreted) with a score of 0.9 and the integral position of the protein was predicted to be membrane-bound chloroplast when observed in ProtComp 9.0 online tool. The InterproScan online tool revealed LCYB to be involved in the carotenoid biosynthetic process with the oxidoreductase activity confirming the protein analyzed belongs to the carotenoid pathway. PSITE tool analysis showed the presence of putative phosphorylation sites such as N-glycosylation, protein kinase C phosphorylation, amidation, N-myristoylation sites, casein kinase II phosphorylation, tyrosine kinase phosphorylation. All these modifications play an important role in salt tolerance in plants suggesting the three proteins to be involved in regulating plant response towards stress (Shao *et al.*, 2018). All the other motif sites are given in Table 8.

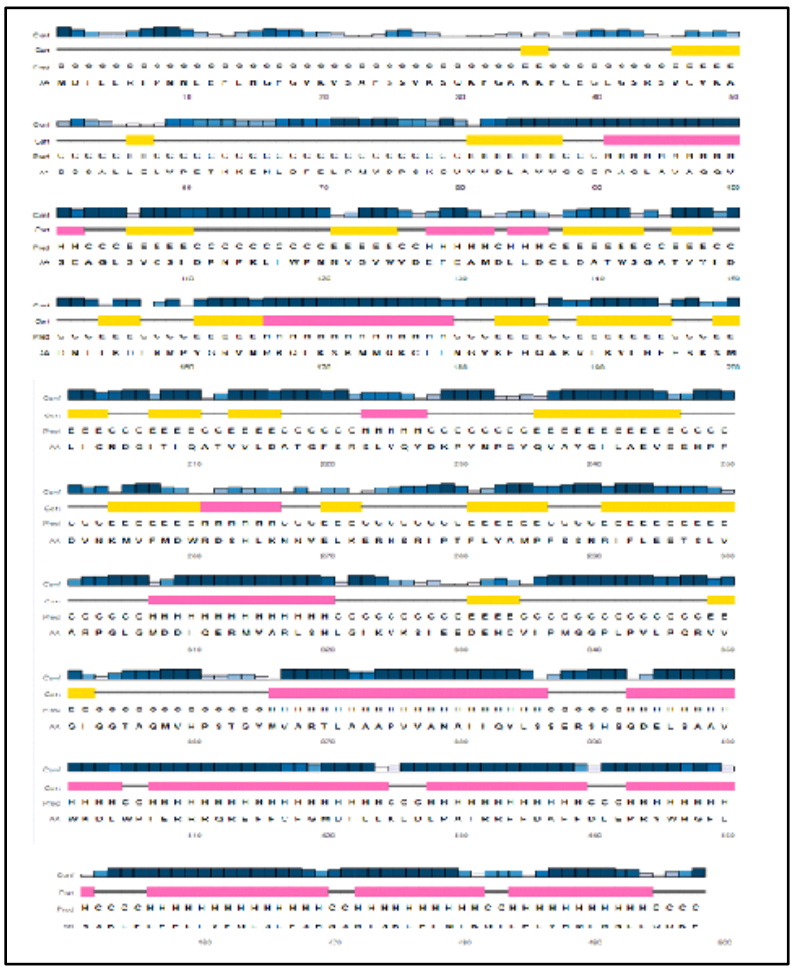


Fig. 6. Secondary structure prediction of lycopene β cyclase protein from *Capsicum frutescens* using Psipred tool

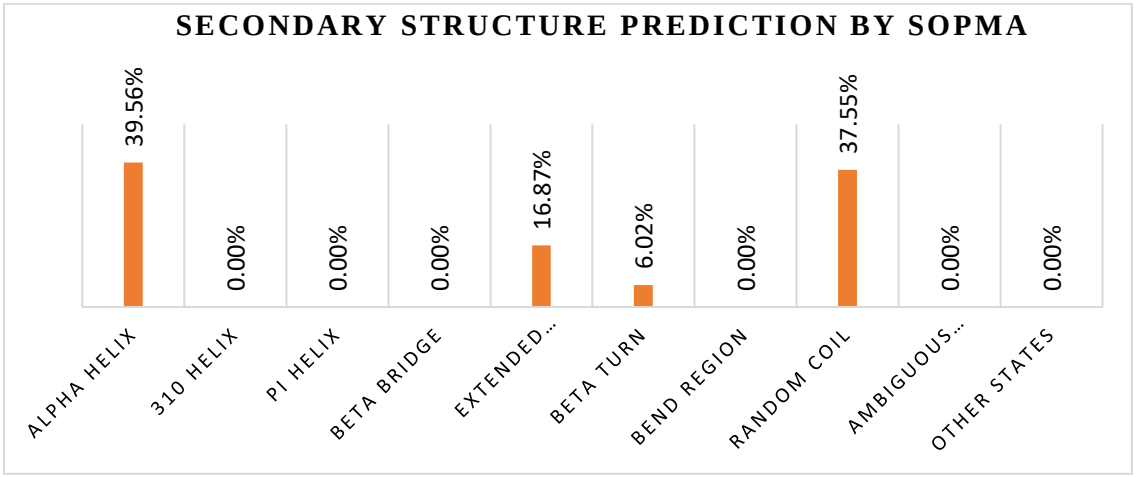


Fig. 7. Secondary structure prediction by SOPMA

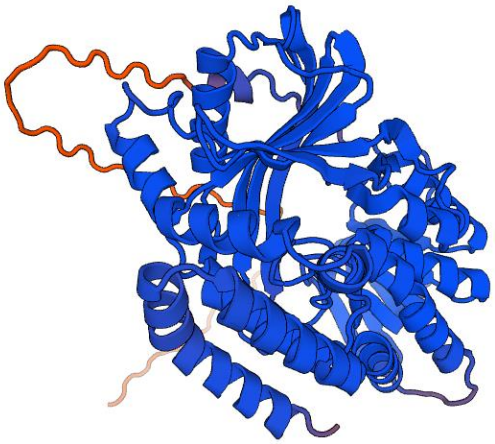


Fig. 8. Tertiary structure analysis of lycopene β cyclase using SWISS model software

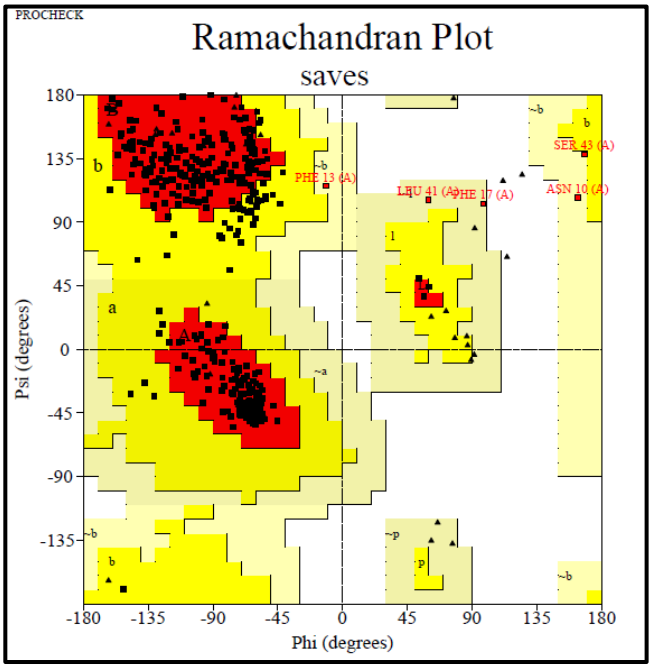


Fig. 9. Ramachandran map of lycopene β cyclase protein from *Capsicum frutescens*

Table 7. Ramachandran plot statistics for lycopene β cyclase

Plot statistics	No. of residues	%
Most favoured regions [A, B, L]	384	88.1
Additional allowed regions [a, b, l, p]	47	10.8
Generously allowed regions [~a, ~b, ~l, ~p]	5	1.1
Disallowed regions	0	0.0
Non-glycine and non-proline residues	436	100.0
End-residues excl. (Gly and Pro)	2	
Glycine residues	34	
Proline residues	26	
Total number of residues	498	

Table 8. Motifs present in the lycopene β cyclase protein of *Capsicum frutescens*

Sl. No.	Motif	Number
1	N-glycosylation site	2
3	cAMP- and cGMP-dependent protein kinase phosphorylation site	-
4	Protein kinase C phosphorylation site	9
5	Casein kinase II phosphorylation site	5
6	Tyrosine kinase phosphorylation site	-
7	N-myristoylation site	7
8	Amidation site	-
9	Prenyl group binding site (CAAX box)	3
10	Microbodies C-terminal targeting signal	12
11	Endoplasmic reticulum targeting sequence	-
12	Ribosomal protein S2 signature 1	-
13	Squalene and phytoene synthases signature 1	-
14	Squalene and phytoene synthases signature 2	-

Conclusion

Capsicum frutescens is one of the most important spices used all over the world traditionally. Most of the research conducted on it focuses on its capsaicinoid level and not on carotenoid content which leaves the enzymes involved in the carotenoid biosynthetic pathway unexplored from this plant. Lycopene β cyclase is the enzyme involved in the conversion of lycopene to β carotene which is an important pharmaceutical molecule, and much research is carried out in improving its content in plants. In the present study, the gene lycopene β cyclase related to carotenoid biosynthesis was isolated from *Capsicum*

frutescens and characterized using different online tools. The study was able to confirm the conserved nature of the gene and the related protein. By analyzing its molecular structure and evolutionary relationship, we have expanded our understanding of the regulatory mechanism of lycopene β cyclase.

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