African Journal of Biotechnology Vol. 10(32), pp. 5934-5939, 4 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2584
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Cloning and heterologous expression of a gene encoding lycopene-epsilon-cyclase, a precursor of lutein in tea (*Camellia sinensis* var *assamica*)

Sangeeta Borchetia*, Chiranjana Bora, Bornali Gohain, Prasenjit Bhagawati, Niraj Agarwala, Neelakshi Bhattacharya, Raju Bharalee, Priyadarshini Bhorali, Tirthankar Bandyopadhyay, Sushmita Gupta, Sourabh Kumar Das, H. Ranjit Singh, Parveen Ahmed, Madhurjya Gogoi and Sudripta Das

Department of Biotechnology and Bioinformatics, Tocklai Experimental Station, Tea Research Association, Jorhat, Assam, India.

Accepted 6 April, 2011

This report describes the cloning and expression of a gene lycopene epsilon cyclase, (LCYE) from *Camellia sinensis* var *assamica* which is a precursor of the carotenoid lutein in tea. The 1982 bp cDNA sequence with 1599 bp open reading frame of LCYE was identified from an SSH library constructed for quality trait in tea. 5' and 3' RACE (rapid-amplification of cDNA ends) was done to clone the full length cDNA of LCYE. Homology studies showed that the deduced amino acid sequence of LCYE gene had the highest sequence identity of up to 84% with *Vitis vinefera*. The cloned gene was successfully expressed in a PET based *Escherichia coli* expression system. The size of the expressed protein was 59615 Daltons. A suppression subtractive library was constructed using a quality clone H3111 (tester) and a garden series clone T3E3 (driver).

Key words: Carotenoid, RACE, heterologous expression, lutein, tea.

INTRODUCTION

Lycopene epsilon cyclase (LCYE) is a key enzyme in the lutein synthesis pathway and catalyses linear lycopene to form cyclic epsilon-carotene, a precursor of lutein. Carotenoids with cyclic end groups are mainly associated with the accessory light harvesting chlorophyll antenna system and serve as antioxidants and light harvesting pigments in plants (Siefermann-Harms, 1987). Carotenoid biosynthesis begins with the formation of phytoene synthase from geranyl geranyl di phosphate phytoene synthase (PSY). Then, phytoene desaturase (PDS) and f-carotene desaturase (ZDS) catalyse the four consecutive desaturation steps to convert phytoene into lycopene (Cunningham et al., 1994). The cyclization of lycopene is a pivotal branching point in this pathway, yielding a-carotene (lutein) with one e-ring and one b-ring, and b-carotene with two b-rings, in which two cyclases, namely lycopene-e-cyclase (LCYE) and lycopene-b-cyclase (LCYb), respectively, are responsible for these reactions. The lycopene e-cyclase which divert the pathway to compounds which are lower in provitamin A value, are other wise of high pharmaceutical value in lutein, a hydroxycarotenoid constituting the macular pigment of the human retina (Olsen, 1989). Increasing lutein intake from foods could increase the density of this pigment and decrease the risk of developing macular degeneration. It is an antioxidant that protects our cells against damage caused by dangerous naturally occurring chemicals known as free radicals.

Beta-carotene, lutein, violaxanthin and neoxanthin are the four basic chloroplast carotenoid pigments isolated and identified in tea plant. It has been estimated that 10% of the total volatile compounds identified in tea aroma is derived from carotenoids and its degradation products (Hazarika and Mahanta, 1983). So, a suppression subtractive library (SSH) was constructed to identify

*Corresponding author. E-mail: sangeeta_b123@sify.com. Tel: 0376-2360973 or 919435705469. Fax: 91-0376-2360474.

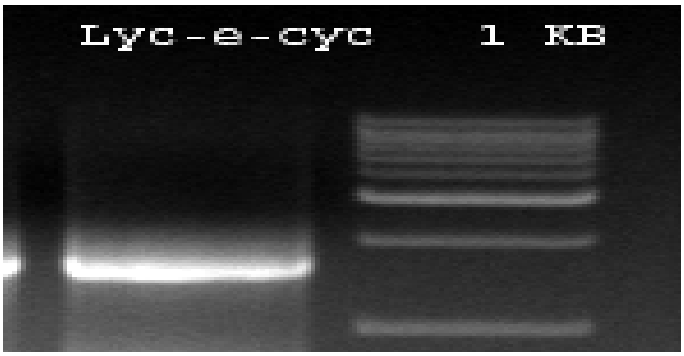

Figure 1. Full length PCR amplification of LYCE of tea (1599 bp).

previously unknown transcripts with tea showing significant homology in various pathways of monoterpenoids, carotenoids, flavonoids and flavour encoding genes in other crops. EST GH710785.1 submitted to the NCBI from the library showed significant homology with lycopene epsilon cyclase in other crops like *Diospyros kaki, Vitis vinifera, Ricinus communis, Citrus limon, Solanum lycopersicum* and *Lycopersicon esculentum*. RNA ligase mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE) was done on this sequence to clone the full length of the gene. The gene was expressed in a heterologous system to analyze those aspects that could give information on the functionality of the protein in a foreign host.

MATERIALS AND METHODS

A suppression subtractive library was constructed using a quality clone H3111 (tester) and a garden series clone T3E3 (driver). Total RNA was isolated from these clones using RNAqueous Kit (Ambion) and mRNA was isolated using PolyATtract mRNA Isolation Systems (Promega, USA). SSH library was prepared using the Clontech PCR-Select cDNA subtraction kit which utilized the SS hybridization technique. The differentially expressed cDNA clones were ligated into pGEMT vector system (Promega) and were transformed using electrocompetent DH10β *Escherichia coli* cells. Plasmids were extracted from the recombinant colonies using Hipura plasmid Miniprep purification spin kit (Hi media) and sequenced using M13F primers in 3130 XL Genetic analyzer (Applied Biosystems).

RACE

Total RNA was isolated from 100 mg of frozen, ground tissue of *Camellia assamica* leaves using 1 ml of TRI-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturers' protocol. The 5' and 3' RACE were performed using GeneRacer Kit (Invitrogen) in accordance with the manufacturers' protocols. Gene specific primers, two forward-primers (5'GAGCAGCTTCGGGGAAACTT3', 5'CCATGCCTATGTCCCCAACA3') and two reverse-primers (5'TGTAGCAAGCCTGCATGGAA3', 5'AGCCTGCATGGAACAACGAC3') were designed to amplify the 5' and 3' of the cDNA ends. The RACE cDNA was amplified using PCR conditions in the Gene Amp PCR System 2720 (AppliedBiosystems) which were as follows: initial denaturation at 98°C for 2 min followed by 35 cycles with 98°C for 30 s, 65°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min. The 5' and 3' products were cloned into Zero Blunt TOPO PCR cloning Kit (Invitrogen) and were transformed into chemically competent DH10β *E. coli* cells. The cloned RACE products were fully sequenced in both directions using M13F primer.

1599 bp LYCE gene was amplified with vector specific inserts and then ligated to Ek/LIC vector (Novagen) by T_4DNA polymerase and was transformed into BL21(DE3)pLysS *E. coli* strain. Overnight cultures of BL21(DE3)pLysS harboring pET-43.1 Ek/LIC plasmid with the LYCE insert was diluted to 10% (vol/vol) in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml), and then were grown aerobically at 37°C. When the optical density at 600 nm reached 0.6, the gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG)(sigma) to a concentration of 0.5, 1 and 1.5 mM for 6 and 9 h. A control was taken with no induction. The cells were harvested by centrifugation at 8000 xg, for 5 min at 4°C and run on a sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel.

RESULTS AND DISCUSSION

The cDNA sequence encoding lycopene epsilon cyclase (LCYE) in *C. sinensis* var *assamica* was 1599 bp in length (Figure 1). A 5' untranslated region of 227 bp preceded the putative start codon and 135 bp of 3' untranslated region followed the termination codon and preceded the poly A tail (Gene Bank accession no. HM536196.1). The predicted amino acid sequence of LCYE specified a polypeptide of 532 amino acid in length with a molecular weight of 59615 Dalton and a pI of 6.54 (Figure 2). The predicted amino acid sequence of *Camellia* was quite similar to those of other known epsilon cyclases such as V*itis, Citrus, Daucus, Ricinnus, Arabidopsis*, etc. Figure 3 is graphically shown to illustrate the degree of similarity and location of highly conserved regions with the predicted amino acid sequence of five related plant species.

The highest similarity of 84% with 80% query coverage was observed with *V. vinifera.* The online softwares such as PredictProtein and Swiss-Model were used in the bioinformatics analysis of the amino acid sequence of LCYE protein. The phylogenetic tree showed the closest relationship between *V. vinifera*, while LCYE of *Chlamydomonas reinhardtii* and lycopene β-cyclase of *Ostreococcus lucimarinus,* which is an early diverging class within the unicellular green plant lineage was seen as an outgroup with about 40% similarity with the LCYE of tea (Figure 4). An amino acid sequence signature indicative of a dinucleotide binding motif which is present in all known carotenoid cyclase was observed from amino acid 109 to 138 which is a characteristic p sheet-a helix-loop-p sheet configuration referred to as the Rossmann fold (Rossman et al., 1974). This sequence signature resembles that found at the N terminus of enzyme that binds FAD. Moreover, from 123 to 499 FAD/NAD(P), binding domain was observed using Swiss Mode Work Space. Various binding motifs such as 2Fe-2S

MECIGARNFAAMAVSTCPIWRYRKKRQRNTLTRVIHRHSSYTSSLRVRSSSAGSESCVAFEEGFADEEDYIKAG

GSELLFVQMQQNKLMEKQSKLADKLPPISVGNTILDLVVIGCGPAGLALAAESAKLGLSVGLIGPDLPFTNNYGV

Di nucleotide binding signature

WEDEFRDLGLEGCIEHVWGDTIVYLDDNDSPFLIGPCFIGRVSRYLLHEELVKRCVESGVSYLSSKVERIIESAIGH

SLIECEQNVVVPCRLATVASGAASGKLLQYEVGGPRVSVQTAYGMEVEVENNPYDPNLMVFMDYRDYVKPR

VQCLEAQYPTFLYAMPMSPTRVFFEETCLASKDAMPFDLLKKKMSRLETMGVRVIKTYEEEWSYIPVGGSLPNT

Cyclase motif 1

Cyclase motif 2

EQKNLALGAAASMVHPATGYFVVRSLSEAPKYASVIANILKQGHSRDKLSRSWSTENISMLAWNTLWPQERK

RQRAFFLFGLALILQLDIDGIRTFFHTFFRLPTWMWQGFLGSTLSSADLALFAFYMFVIAPNNMRMCLVRHLLS

DPTGATMIRTYLTI*

Figure 2. Predicted 532 amino acid sequence of LYCE of tea with cyclase motifs and Rossmann fold of carotenoid di nucleotide binding signature. The sequence of the full length of LYCE gene cloned and sequenced was deposited in NCBI database under accession No. GenBank HM536196.1.

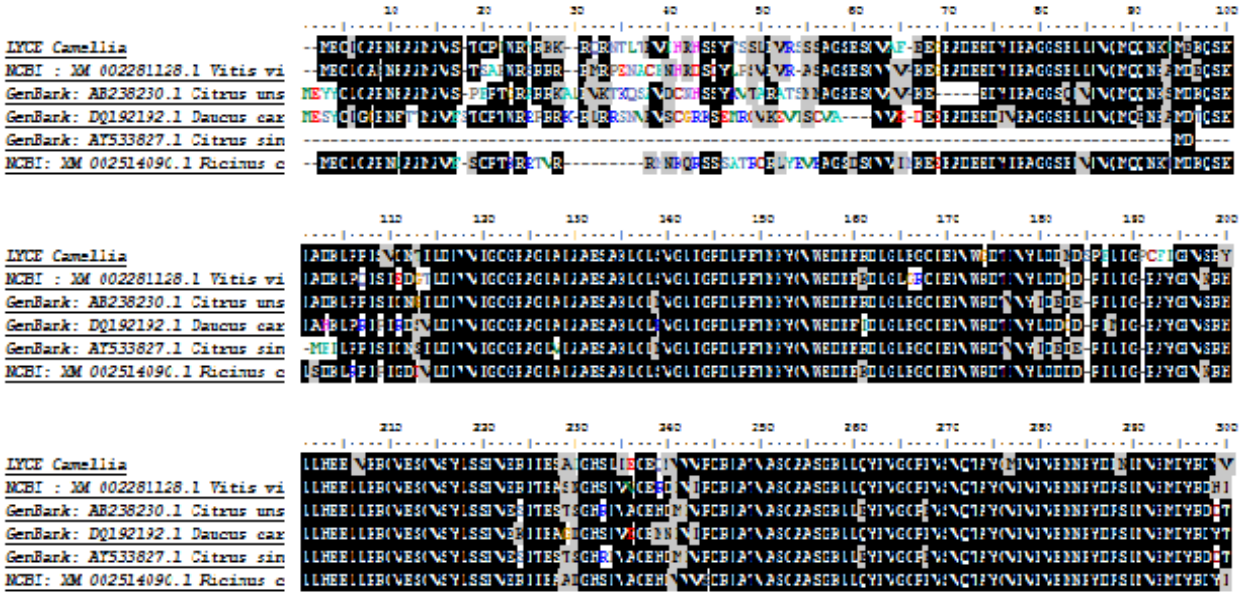

Figure 3. Alignment of the deduced amino acid sequence of Lyc-e-cyclase with homologous sequences from *Vitis vinifera* (XM_002281128.1), *Citrus unshiu* (AB238230.1), *Daucus carota* (DQ192192.1), *Citrus sinensis* (AY533827.1) and *Ricinus communis*(XM_002514090.1). Amino acids identical in at least 3 are highlighted in black. Gaps are introduced to maximize the alignment. The sequence obtained was analyzed using NCBI Blast for sequence homology.

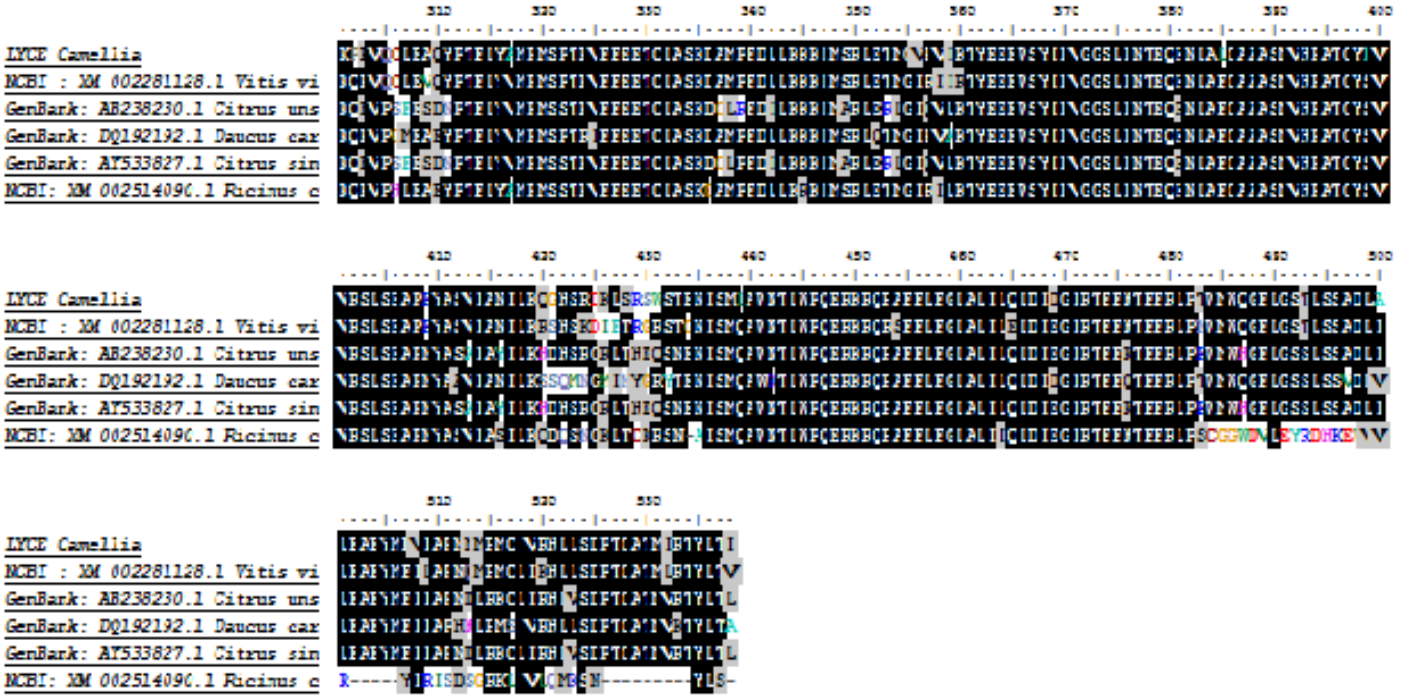

Figure 3. Contd.

ferredoxins or 4Fe-4S ferredoxins, iron-sulfur binding region signature, *N*-glycosylation site, protein kinase C phosphorylation site, casein kinase II phosphorylation site, tyrosine kinase phosphorylation site and *N*-myristoylation site were found in LCYE cDNA of tea using database search. Moreover, two cyclase motifs were observed between the 306th and 322th, and the 382th and 391th positions of the sequence. The predicted amino acid composition of tea LCYE was hydrophobic with a higher percentage of hydrophobic and nonpolar amino acids. Plots of the hydropathic index of LCYE of tea contained several hydrophobic regions that would correspond to the membrane spanning helical regions and indicated a high probability of forming a transmembrane helix as a region showed a hydrophobicity value greater than 1.6 in a window of 19 amino acids, according to Kyte and Doolittle (1982). A higher aliphatic index of 90.36 indicates that the predicted protein is thermostable. The functional state of the protein was successfully expressed in BL21(DE3)pLysS *E. coli* strain by directional cloning of PCR-amplifed LCYE gene sequence in pET-43.1 Ek/LIC plasmids, driven by the strong T7 bacteriophage promoter which was transcribed actively and selectively under the control of the T7 RNA polymerase. This system was able to maintain the target genes transcriptionally silent in the uninduced state. However, upon induction with 1.5 mM IPTG for 6 h (Figure 5), the λDE3 lysogens with LCYE insert was highly induced. The LCYE protein was found to be a potentially active enzyme as it was correctly expressed and functionally processed in a heterologous system.

ACKNOWLEDGEMENTS

The authors acknowledge the generous funding of DBT, by the Government of India and also the encouragement and support of the Director of TRA.

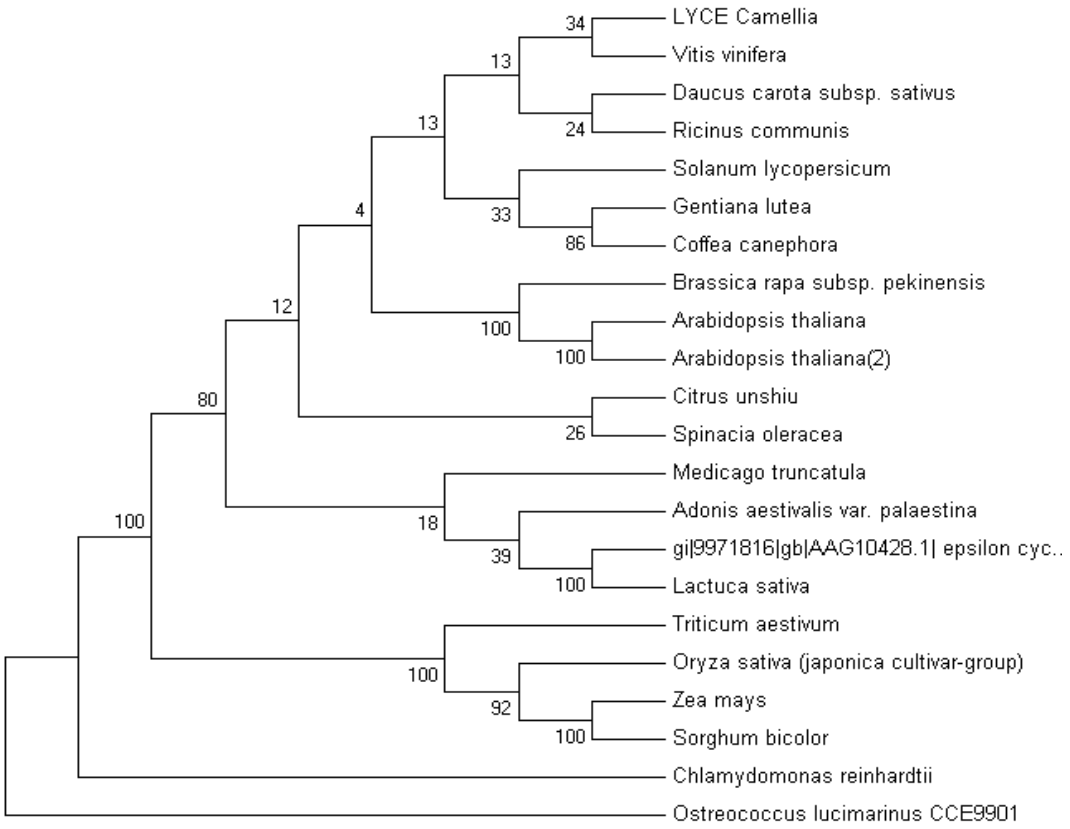

Figure 4. A phylogenetic tree view of the related species with LYCE of tea. The bootstrap cladogram was drawn by the Neighbor Joining method.

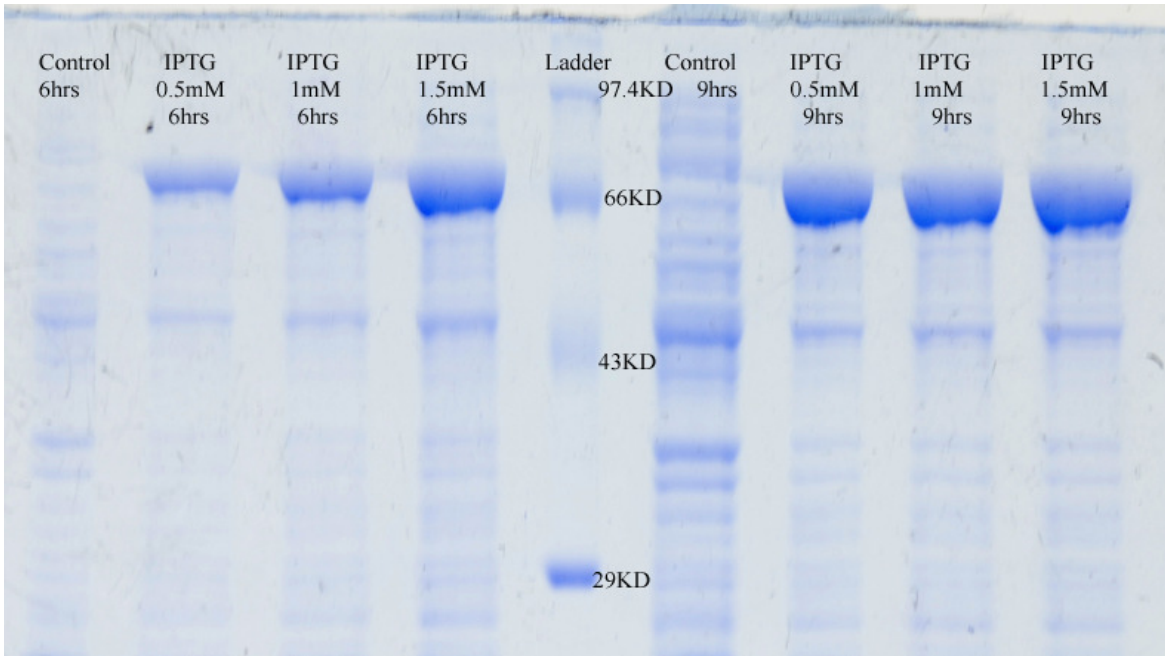

Figure 5. Expression analysis of LYCE protein in BL21 (DE3)pLysS *E. coli* strain carrying the recombinant plasmid in Coomasie Blue-stained SDS-12.5% polyacrylamide gels. Lanes 1 and 6 consistsof uninduced total protein in 6 and 9 h; lanes 2, 3 and 4 consisted of 6 h induced total protein; lanes 7, 8, 9, consisted of 9 h induced total protein with IPTG. The sizes of the molecular weight markers (Lane 5) are indicated to the right of the bands.

REFERENCES

Cunningham FX, Sun Z, Chamovitz D, Hirschberg J, Gantt E (1994). Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp.strain PCC7942. Plant Cell, 6: 1107–1121.

Hazarika M, Mahanta PK (1983). Some studies on carotenoids and their degradation in black tea manufacture. J. Sci. Food Agric. 34: 1390-1396.

Kyte J, Doolittle RF (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

Olsen JA (1989). Provitamin-A function of carotenoids: the conversion of-carotene into vitamin-A. J. Nutr. 119: 105-108.

Rossmann MG, Moras D, Olsen KW (1974). Chemical and biological evolution of a nucleotide-binding fold. Nature, 250: 194-199.

Siefermann-Harms D (1987). The light-harvesting and protective functions of carotenoids in photosynthetic membranes. German Physiol. Plant. 69: 561-568.