

Comparative Analysis of Lycopene Gene in Green and Purple Coloured Brassica Oleracea Varieties

T Balasaravanan*, Juby Elsa Joseph

Department of Biotechnology, Nehru Arts and Science College, Bharathiar University, Coimbatore, India

Research Article

Received date: 03/06/2021
Accepted date: 17/06/2021
Published date: 31/08/2021

*For Correspondence

T Balasaravanan, Department of Biotechnology, Nehru Arts and Science College, Bharathiar University, Coimbatore, India.

E-mail: balsarvan@rediffmail.com

Keywords: Brassica oleracea; Carotenoid; Lycopene; Homology modelling; Phylogenetic analysis

ABSTRACT

The carotenoid pigmented fruits and vegetables have high nutritional values as they are rich in vitamin A and antioxidant properties. There are numerous coloured fruits and vegetables and plenty of researches have been done on them determining their gene expression levels in different species and even at different parts of a plant as well. The present study aimed the comparative analysis of lycopene genes in purple colour cabbage and green colour cabbage. The mRNA from both green and purple cabbage leaves were isolated and converted to cDNA. Then their lycopene cDNAs were amplified with suitable set of primers and these were then sequenced. The mutations observed in both the sequences were negligibly small. So, the carotenoid pigment will be due to some other factor. From their DNA sequences, amino acid sequences were obtained, and homology modelling provided suitable structure for the proteins obtained from both green and purple coloured cabbage. Then phylogenetic analysis was done with different lycopene amino acid sequences of species like tomato, papaya, corn, citrus and Arabidopsis determined that the isolated lycopene gene from the cabbage varieties are more similar to lycopene β -2 cyclase gene (LCY-B2). From this study, it can be concluded that lycopene is present not just in coloured vegetables but also in green leafy vegetable like cabbage. According to the spectrophotometric determination of lycopene content in both varieties of cabbage, it was found that purple coloured cabbage leaves have more lycopene content than that of green leafy cabbage.

INTRODUCTION

Fruits and vegetable-based diets should be promoted in all periods of life. They are an important part of our diet and exert benefits on our health becoming more evident every day. While determining the dietary significance of a food, often nutrients are the only elements considered, although other less known components called 'non-nutrients' are also of huge interest regarding health. At times they have important physiological properties and are therefore considered as bioactive substances. Such substances when found in plants are known as "phytochemicals". The phytochemicals stand out among 'non-nutrients' because, in addition to having beneficial properties, they give fruits and vegetables their colour. In Camera's research work it was also found that green leafy vegetables contain high amounts of ascorbic acid or vitamin C, iron and folic acid [1].

Broccoli, Brussel sprouts, cauliflower and Brassica oleracea contain glucosinolates. Heber and Bowerman also stated that the red colour of vegetables and fruits such as tomato and watermelon is due to a compound called lycopene, which also belongs to the group of carotenoids, just like α and β -carotene. While the red-purple colour of grapefruits berries, raspberries and cranberries are caused by anthocyanins, the orange colour in vegetables and fruits such as carrot, mango, pumpkin, are due to phytochemicals or the carotenoids like α , γ , β -carotene. Also, flavonoids together with β -cryptoxanthin are responsible for the light orange to yellow colour of fruits such as papaya, peach and orange. α and β -carotene are especially inevitable in the diet because they are vitamin A precursors, which is why they are also known as provitamin A. Vitamin A is involved in hormone synthesis, regulation of immune responses as well as cell growth and differentiation. The β -cryptoxanthin like β -carotene or lycopene is also a carotenoid, and has an important function as biological antioxidant, protecting different tissues and cells from oxidative damage [2].

During the synthesis of a family of carotenoids, lycopene is one of the carotenoids that are produced first, thus constitutes the base to synthesize the others. Lycopene is not a vitamin A precursor, except α - and β -carotene. Lycopene is a carotenoid with simple aliphatic chain structure of 40 carbon atoms and many conjugated double bonds. Many research states that lycopene acts as a very strong antioxidant and plays an important role in cell communication. There are some experimental evidences that prove its protective function against cardiovascular disease, prostate cancer and damage from ultraviolet light exposure

and tobacco smoke. Anthocyanins belong to the biggest group of phenolic compounds, called flavonoids. It has been discovered that anthocyanins are absorbed from the diet directly without being modified. In several in vivo and in vitro experiments, it was demonstrated that anthocyanins have antioxidant properties and plays an important role in preventing carcinogenesis and mutagenesis.

Carotenoid biosynthesis begins with the formation of phytoene from geranylgeranyl diphosphate. Carotenoid pigments are indispensable for photosynthesis in plants. The carotenoid biosynthesis pathway has been extensively studied in many photosynthetic and non-photosynthetic organisms. In many species, carotenoids can also accumulate as secondary metabolites in chromoplasts of flowers, fruits, seeds or roots to provide distinct coloration, ranging from yellow to orange and red. Lycopene is a precursor of β -carotene and β -cryptoxanthin. In tomato, lycopene accumulation during fruit ripening is due to the down regulation of lycopene β -cyclase activity. The fruit flesh colour of papaya is determined largely by the carotenoid content. Red-fleshed papaya fruit contain high levels of lycopene, whereas yellow-fleshed fruit do not. The two major papaya fruit flesh colours, red and yellow, are controlled by a single genetic locus with yellow being dominant over red [3].

Most previous research demonstrated that the preliminary mechanism that controls lycopene aggregation in tomato fruits is based on the distinct regulation of expression of carotenoid biosynthesis genes. At the fruit development stage, the mRNA levels for the lycopene-producing enzymes like Phytoene Desaturase (PDS) and phytoene synthase increase. At the same time the mRNA levels of the genes coding for lycopene ϵ - and β -cyclases that convert lycopene to either δ - or β -carotene respectively, later decline and finally completely disappear. The mechanism in papaya is a little different from that of tomato fruits. Some studies found that the colour of papaya fruit flesh is determined primarily by the presence of carotenoid pigments. Lycopene is present in red-fleshed papaya fruit, while this pigment is absent in yellow-fleshed papaya fruit. The conversion of lycopene to β -carotene is catalyzed by lycopene β -cyclase. There are two different genes that encode lycopene β -cyclases namely LCY- β 1 and LCY- β 2. A change in the LCY- β 2 gene inactivates enzyme activity that controls lycopene production in the fruit and is thus responsible for the difference in carotenoid production between red and yellow-fleshed papaya fruit. LCY- β 1 and LCY- β 2 genes have similar expression levels but have comparatively low levels in leaves. But the LCY- β 2 expression increases remarkably in ripe fruit. The reason behind conducting research on Brassica oleracea variety was to understand if the lycopene content is influenced by or limited only to ripened fruit or not. This study aims at a comparative study on lycopene expression level in green and purple colored vegetable (Brassica oleracea) instead of red colored fruits [4].

MATERIALS AND METHODS

Standardization of protocol for mRNA isolation from cabbage leaves

For RNA isolation from green colour cabbage and purple colour cabbage, two different methods was used. One was RNA isolation method, with TRIzol reagent. The surface sterilized leaf tissues of both the varieties (each 100 mg) were finely grinded with mortar and pestle along with TRIzol reagent, incubated at room temperature for 5 min. The concoction transferred to a fresh eppendorf tube and added 0.1 ml chloroform. After proper mixing and incubation at room temperature for 2-5 min, it is centrifuged at 10,000 rpm for 15 minutes at 20C-50C. Then, colorless aqueous phase was collected in another eppendorf, added 2-propanol (0.5 ml), mixed well and allowed to stand for 5-10 min at room temperature. Pelletized by centrifuging at 10,000 rpm for 10 min at 20C-80C, 75% ethanol (0.5 ml) was added and vortexed. Again, samples were centrifuged at 8,000 rpm for 5 min and the pellet was air dried then suspended in 100 μ l of nuclease free water.

Another method used followed a different protocol. The surface sterilized leaf samples (each 100 mg) were ground finely with a pinch of PVP along with β -mercaptoethanol (20 μ l) and RNA extraction buffer (1 ml) with the help of motor and pestle. The mixture of each sample was then transferred to individual eppendorf tubes and centrifuged at 8000 rpm for 5 min. Supernatant was transferred to another fresh tube and equal volume of chloroform was added. They were then kept in ice for 5 min after mixing properly. Then centrifuged at 8000 rpm for 10 min and the supernatant were transferred to another new eppendorf tube. An equal volume of isopropanol was added and kept in ice for few minutes. Then centrifuged at 8000 rpm for 5 min and the supernatant were discarded. Added 70% ethanol to the pellet and washed, centrifuged at 8000 rpm for 5 min. The ethanol supernatant discarded, air dried the pellet and suspended in 100 μ l of nuclease free water. Then the samples were loaded on a 1% agarose gel in TBE buffer and electrophoresis was done to determine the presence of RNA [5].

DNase treatment of the obtained RNA sample

From the RNA samples, obtained from both green and purple colored cabbage, 10 μ l each was taken to a fresh eppendorf tube. Then RNase free DNase (1 μ l) was added and kept in incubation at 37oC for 30 min to degrade DNA present in the RNA samples. Added equal volume of chloroform mixed and centrifuged at 12,000 g for 15 min. The supernatants were collected in another tube and 2.5 volume of ice-cold absolute ethanol was added to it. The samples were mixed and kept at room temperature for 5 min. Then it was centrifuged at 12,000 g for 10 min. The supernatant was discarded, the pellet was air dried and was dissolved in nuclease free water. Then run on 1% agarose gel electrophoresis.

mRNA converted to c-DNA

The obtained template RNA sample (2 μ l) along with oligo dT primer (1 μ l), 10 mM dNTP mix (1 μ l), 5X reverse transcriptase buffer (4 μ l), enzyme mix (1 μ l) were taken in a PCR tube and the volume was made up to a total of 20 μ l with nuclease free water. This was mixed thoroughly and incubated at 50oC for 30 min and then at 65oC for 5 min.

Primer designing for lycopene c-DNA amplification

c-DNA sequences of lycopene β -cyclase of navel orange, capsicum, tomato, watermelon and papaya were obtained using the Basic Local Alignment Search Tool (BLAST) available at National Centre for Biotechnology Information (NCBI). All LCY-B sequences were aligned using the multiple sequence alignment tool Clustal Omega to produce a consensus sequence. A 25 bp forward-primer and 20 bp reverse-primer were designed. The primers were checked for dimer and hairpin formation using the software Oligoanalyzer 3.0 from Integrated DNA Technologies Inc. From the obtained multiple sequences, forward and backward primers were designed with Primer 3 software [6].

Estimation of c-DNA sample concentration

With the help of UV-Visible spectroscopy, the concentration of the obtained c-DNA of green and purple colored cabbage was estimated. Keeping blank as PCR water, concentration of each samples was determined. The c-DNA samples (1 μ l each) of both green and purple cabbage were taken in different cuvettes, and then diluted with PCR water (999 μ l) and concentration was measured. Thus, the concentration of the c-DNA of green colored cabbage was found to be 0.042 ng/ μ l and that of purple colored cabbage was 0.063 ng/ μ l. The obtained concentrations were converted in terms of 100 ng/ μ l for dilution.

Synthesized c-DNA is amplified by RT-PCR

PCR amplification was done in 25 μ l reactions each consisting of PCR water (17.7 μ l), Taq buffer (2.5 μ l), MgCl₂ (1 μ l), dNTPs mix (0.5 μ l), Taq DNA polymerase (0.3 μ l), forward primer- 5' GCTCTCTGTGTAGGAAGATAATAGT 3' (0.5 μ l), reverse primer- 5' TAGCGTCCAGAACACAGAA 3' (0.5 μ l) and diluted template c-DNA (2 μ l). Thermo cycler conditions consisted of an initial 3 min denaturation at 95°C, followed by 30 cycles of annealing at 60°C for 30 sec, elongation for 2 min at 72°C and a final extension for 10 min.

DNA Sequencing and analysis of DNA sequences

DNA sequences of c-DNA PCR product of both green and purple cabbage were obtained. The sequence differences or mutations were analyzed with the help of pairwise sequence alignment tool in the Clustal software.

Homology modelling

The DNA sequences were converted to protein sequences with the help of ExPasy software. Keeping these protein sequences as target sequences, lycopene protein sequence 1 VHQ was found from Protein Data Bank (PDB), which was taken as template sequence. Then with the help of Swiss-Model tool, homology models were built.

Phylogenetic analysis

Phylogenetic analysis was done by multiple alignments of conceptual amino acid sequences of various lycopene β cyclase genes obtained from GenBank. Multiple alignments of protein sequences from papaya LCY-B1, papaya LCY-B2, Arabidopsis LCY-B, tomato LCY-B1, tomato LCY-B2 along with the protein sequences obtained for both green colored cabbage and purple colored cabbages [7].

Spectrophotometric determination of lycopene

Well homogenized cabbage juices from both green and purple colored cabbage was prepared and a 100 μ l of the sample was taken into a pipette. Then dispensed these samples into screw cap tubes and added 8.0 ml of hexane: ethanol: acetone (2:1:1) into the screw cap tubes having sample. The tubes were then tightened and mixed immediately. Then it was kept in dark for few hours and added 1.0 ml water to each sample and vortexed thoroughly. The samples were allowed to stand for 10 minutes so that the phases get separated and all air bubbles disappear. Further, with the help of spectrophotometer at 503 nm, the lycopene content in the samples were determined.

RESULTS AND DISCUSSION

m-RNA isolation from cabbage leaves

The RNA isolation of green and purple colored cabbage was optimized and by following the second method, good quality RNA samples were isolated with a little presence of DNA in it. Then the DNA was removed by DNase treatment. This was then converted to c-DNA. With the help of UV-Visible spectroscopy, the concentration of the obtained c-DNA of green and purple colored cabbage was estimated. The concentration of the c-DNA of green colored cabbage was found to be 0.042 ng/ μ l and that of purple colored cabbage was 0.063 ng/ μ l, these concentrations were converted in terms of 100 ng/ μ l for dilution. In earlier research, the total RNA was extracted from leaves, flowers and fruits of various papaya cultivars for c-DNA synthesis, which was done with TRIzol reagent.

c-DNA amplification by RT-PCR

The diluted c-DNA samples were then amplified by Polymerase Chain Reaction (PCR). These amplified c-DNA samples were loaded on 1% agarose gel and electrophoresed, visualized under UV-trans illuminator. Clear PCR amplified DNA bands were obtained for both green colour cabbage as well as purple colour cabbage. But the band colour intensities varied showing a light band for green colored cabbage and a darker band for purple colour cabbage indicating the lycopene gene expression variation in both the

varieties. The PCR amplified c-DNA of both the varieties was sequenced. The sequence differences were analysed with the help of pairwise sequence alignment tool in the Clustal software. Thus, the mutations were found from these DNA sequences (Figure 1) [8].

Homology models

The DNA sequences were then converted to amino acid sequences with the help of ExPASy software. Keeping the obtained protein sequences as target sequences, lycopene protein sequence 1VHQ was found from Protein Data Bank (PDB), which was taken as template sequence. Then with the help of SWISS-MODEL tool, homology models were built for both varieties of cabbage [9-12].

Phylogenetic tree

The phylogenetic analysis was done with the help of Clustal Omega 1.2.1 version from GenBank. Multiple alignment of protein sequences from papaya LCY-B1, papaya LCY-B2, Arabidopsis LCY-B, tomato LCY-B1, tomato LCY-B2 along with the protein sequences obtained for both green and purple colored cabbages were generated using Clustal Omega version 1.2.1. Following this, a complete phylogenetic analysis was carried out (Figure 2) [13]. Research based on carotenoid biosynthesis are extensively done in plants such as tomato, papaya, Arabidopsis and pepper. The amino acid sequences of various ϵ -cyclase and β -lycopene genes were obtained from GenBank in his research works. Then multiple sequence alignment of the amino acid sequences obtained from papaya LCY-B1 (ABD91578), papaya LCY-B2, Arabidopsis LCY-E, Arabidopsis LCY-B, tomato LCY-B1, tomato LCY-B2 and tomato LCY-E were generated using Crustal version 2.0.11. The distances in phylogenetic tree were determined using the p-distance option (Figure 3). The tree was tested by bootstrapping with 1000 replications and inferred using the Neighbor-Joining methodology. The values displayed are percentage consensus at the tree nodes support as determined by the bootstrapping. Thus, a phylogenetic analysis was done using MEGA 4 [14].

From the phylogenetic tree, it can be stated that the evolutionary history of Arabidopsis has some similarity with cabbage (*Brassica oleracea*). According to the obtained phylogenetic tree, it can also be stated that the lycopene β -cyclase genes of Arabidopsis, tomato, papaya and corn have similarities and are different from lycopene β -1 cyclase. Thus, the lycopene sequences obtained from green and purple colored cabbage will be similar to that of lycopene β -2 cyclase (LCY-B2) and maybe the lycopene sequences obtained for Arabidopsis and corn will be also of LCY-B2 not LCY-B1. Numerous research done by different group of scientists have proven that genes coding for enzymes that functions as a catalyzing agent in the main steps of carotenoid biosynthesis pathway have been cloned and their expression studies have also been done in different species. It was found that tomato fruit is a good model system for studying the carotenogenesis in plants and ripening of tomato fruit is associated with bright changes in its colour. The fruit colour change from green to orange and then to red which is accompanied by dislodging in carotenoid profile from β -carotene to lycopene at a red ripe stage. Through different research it was found that these changes are brought out by transcriptional up regulation of Phytoene Desaturase (PDS) and Phytoene Synthase (PSY1) genes and down regulation of Lycopene ϵ -cyclase (CRTL-e) and Lycopene β -cyclase (LCY-B) genes. By genetic engineering of carotenoid biosynthesis in canola, rice, potato and maize, a significant increase in carotenoid content was achieved. While only a very limited success has been achieved in enriching the level of carotenoids present in tomato when compared to that of any transgenic crops. Researchers

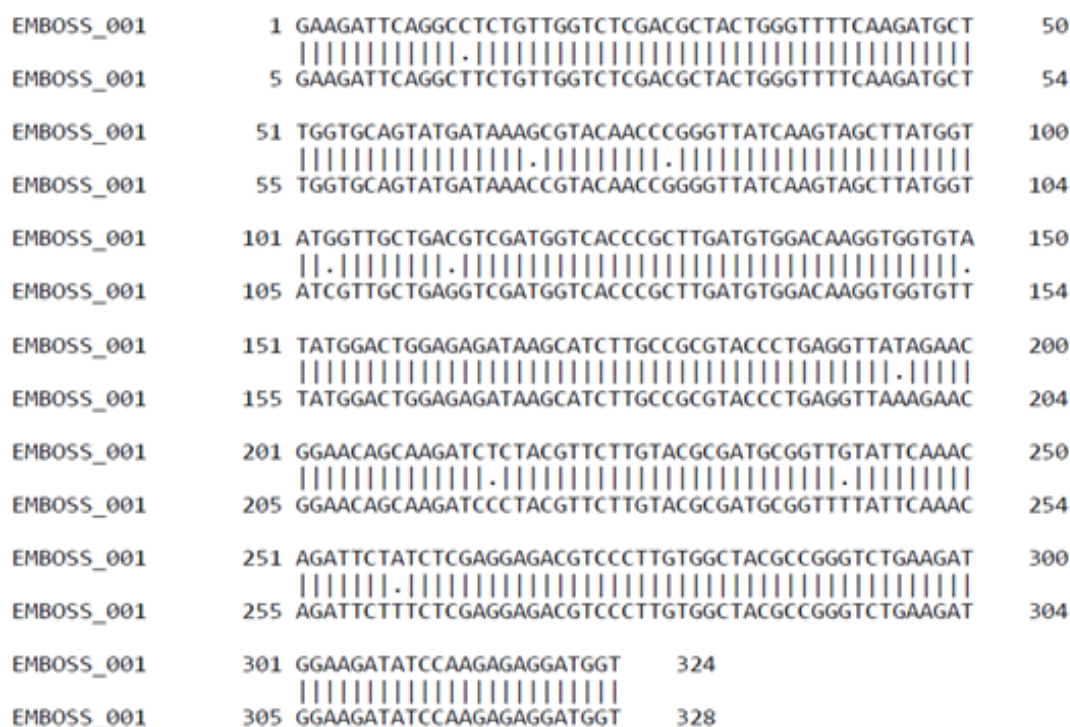


Figure 1: Mutation obtained through Pairwise Sequence Alignment tool.

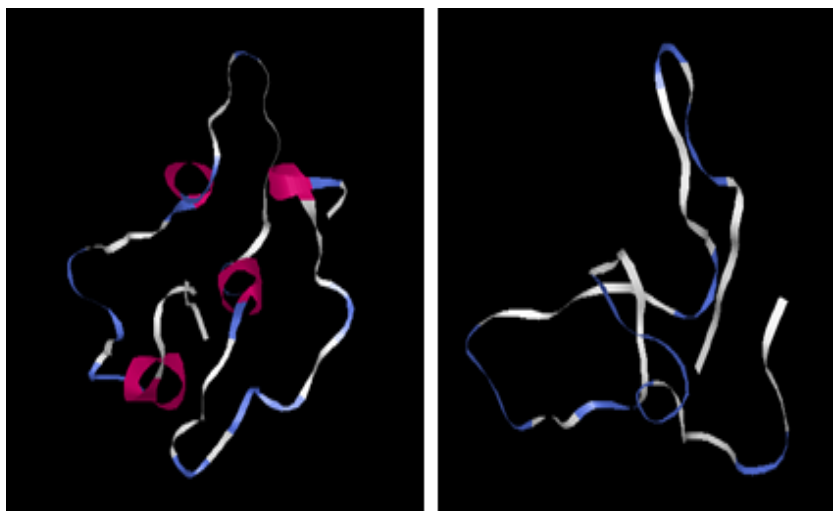


Figure 2: Model of LCY-B of green colored cabbage and purple colored cabbage.

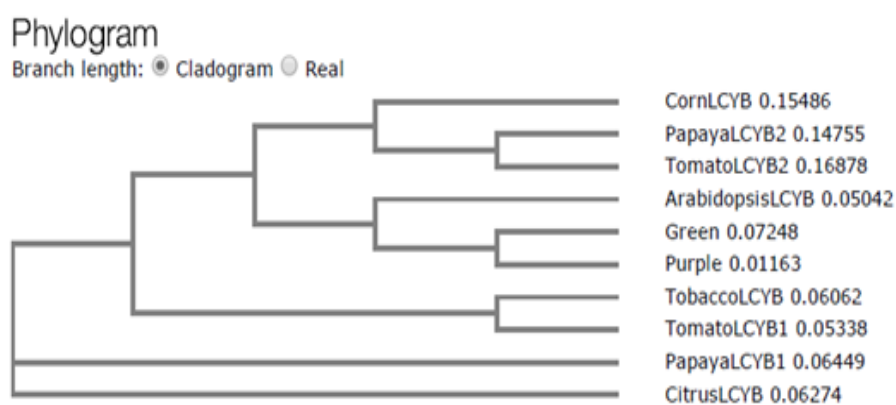


Figure 3: Phylogenetic Tree.

cloned a papaya lycopene β -cyclase, CpLCY- β , the enzyme product which mediates the conversion of lycopene to β -carotene. However, CpLCY- β was not expressed differentially between yellow- and red-fleshed papaya fruit; instead it showed 7-fold higher expression in leaves than in fruit that indicated its role as a chloroplast-specific lycopene β -cyclase [15].

Lycopene content determination

In spectrophotometric analysis, it was observed that the purple colored cabbage leaves have higher lycopene content (3456 microgram/100 ml) than that of green colored cabbage (2112 microgram/100 ml). It was found that even though orange colored fruits and vegetables often have higher carotenoid content and are categorized separately, many dark green vegetables like spinach are also high in carotenoids.

In the present study, I found that lycopene which is also an important carotenoid, is not only present in purple colored cabbage but also present in green colored cabbage as well. World Health Organization and researches have the same opinion that papaya is one of the crops that are especially marked for nutrient enhancement that are to be used in sustainable basic foundation programs to fight against vitamin A deficiency in developing nations. The two major papaya fruit flesh colours like red and yellow are controlled by a single genetic locus with yellow dominant over red. The fruit flesh colour of papaya is evaluated largely by the carotenoid content in them. And the red-fleshed papaya fruit contains high levels of lycopene, whereas yellow-fleshed fruit have only limited lycopene presence. This study proves that carotenoids like lycopene are not only present in colored vegetables, it can also be found in green vegetables as well [16-18].

CONCLUSION

The present research was successful in estimating the presence of lycopene in both green colored as well as purple colored cabbage. It could standardize a proper protocol to isolate the total RNA from the leaves of cabbage and the obtained RNAs were converted to c-DNA. In this work, forward and reverse primers were constructed that could amplify the lycopene c-DNA samples. The c-DNA samples were amplified through Polymerase Chain Reaction (PCR) at specific thermo cycler conditions and were sequenced. The sequenced DNAs were aligned in pairwise sequence alignment tool in Clustal Omega and mutations were recorded. These DNA sequences were converted to protein sequences with the help of ExPASy translate tool. These protein sequences along with the lycopene amino acid sequences of other species like papaya LCY-B1, papaya LCY-B2, Arabidopsis LCY-B, Citrus LCY-B, Tobacco LCY-B, Corn LCY-B, tomato LCY-B1, tomato LCY-B2 were aligned by multiple sequence alignment tools in

Clustal Omega and a phylogenetic analysis was designed. The phylogenetic analysis thus helped in inferring the evolutionary relationship that Arabidopsis and cabbage varieties have some evolutionary similarities and the lycopene gene obtained from the cabbage varieties is similar to LCY-B2 isoform. The spectrophotometric analysis states that the purple colored cabbage leaves have more lycopene content.

REFERENCES

1. Alba R, et al. Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *Plant Cell*. 2005; 17: 2954-65.
2. Aluru M, et al. Generation of transgenic maize with enhanced provitamin content. *J Exp Bot*. 2008; 59: 3551-62.
3. Chandrika UG, et al. Carotenoids in yellow-and red-fleshed papaya (*Carica papaya* L). *J Sci Food Agric*. 2003; 83: 1279-82.
4. McGhie T, et al. The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. *J Exp Bot*. 2009; 60: 3765-79.
5. Davuluri GR, et al. Fruit specific RNAi-mediated suppression of DET1 enhances carotenoid and flavanoid content in tomatoes. *Nat Biotechnol*. 2005; 23: 890-5.
6. Devitt LC, et al. Isolation and functional characterization of a lycopene β -cyclase gene that controls fruit colour of papaya (*Carica papaya* L). *J Exp Bot*. 2010; 61: 33-9.
7. Devitt LC, et al. Discovery of genes associated with fruit ripening in *Carica papaya* using expressed sequence tags. *Plant Sci*. 2006; 170: 356-63.
8. Dharmapuri S, et al. Metabolic engineering of xanthophyll content in tomato fruits. *FEBS Letters*. 2002; 519: 30-4.
9. Ducreux LJM, et al. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. *J Exp Bot*. 2005; 56: 81-9.
10. Ha SW, et al. A comparison of the carotenoid accumulation in *Capsicum* varieties that show different ripening colours: Deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. *J Exp Bot*. 2007; 58: 3135-44.
11. Heber D, et al. Applying science to changing dietary patterns. American institute for cancer research 11th annual research conference on diet. *J Nutr*. 2001; 131: 3078-81.
12. Hirschberg J. Carotenoid biosynthesis in flowering plants. *Curr Opin Plant Biol*. 2001; 4: 210-8.
13. Hounsome N, et al. Plant metabolites and nutritional quality of vegetables. *J Food Sci*. 2008; 73: 48-65.
14. Lazze MC, et al. Anthocyanins induces cell cycle perturbations and apoptosis in different human cell lines. *Carcinogenesis*. 2004; 25: 1427-33.
15. Paine JA, et al. Improving the nutritional value of golden rice through increased pro-vitamin A content. *Nat Biotechnol*. 2005; 23: 482-7.
16. Pecker I, et al. Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Mol Biol*. 1996; 30: 807-19.
17. Romer S, et al. Elevation of the provitamin a content of transgenic tomato plants. *Nat Biotechnol*. 2000; 18: 666-9.
18. Ronen G, et al. An alternative pathway to beta-carotene formation in plant chromoplasts discovered by map-based cloning of beta and old-gold colour mutations in tomato. *Proc Natl Acad Sci*. 2000; 97: 11102-07.