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Original Article

Isolation and Characterization of GDP-L-Galactose Phosphorylase Gene of Vitamin C Biosynthesis Pathway from Kiwi

Soraya Shiri[1], Alireza Zebarjadi[1*]

[1]Campus of Agriculture and Natural Resources, Razi University, Kermanshah, Iran

Corresponding Author: Alireza Zebarjadi, PhD, Associate Professor, Campus of Agriculture and Natural Resources, Razi University, Kermanshah, Iran. Email: Zebarjadiali@yahoo.com

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Abstract

Introduction: Vitamin C is a major antioxidant in plants and plays an important role in reducing the activity of reactive oxygen species. In humans, the main role of this molecule is the elimination of activity of active oxygen species along with being cofactor for many enzymes. Human is one of the few mammalian species that can not synthesize this vitamin and needs to get it through food sources. The GDP-L-galactose phosphorylase (*GGP*) gene is one of the most important genes in the biosynthetic pathway of vitamin C, which codes for the GDPL-galactose phosphorylase enzyme. Isolation of *GGP* gene is an important step in transferring it to elevate vitamin C biosynthesis in plants.

Materials and Methods: In current study, the isolation of this gene from kiwi plant was carried out and then was cloned in the pTG19-T plasmid via T/A cloning and subsequently sequenced to confirm it.

Results: Sequencing analysis of the *GGP* gene showed that this fragment contains 1383 bp and the start and stop codons were ATG and TGA, respectively. The bioinformatics analysis of this gene can provide important information on gene and protein structure. The alignment of cloned sequence was done with other *Actinidia* DNA sequences. The results based on neighbor-joining alignment showed that some of the mutations in nucleotides were related to the third nucleotide in a specific codon. Also, the minimum distance of protein sequences was observed between isolated *GGP* and *Actinidia chinensis*.

Conclusions: Based on analyses, isolated gene (*GGP*) can be used for increase vitamin C content in other plants such as cucumber and for resistance to environmental stresses in different plants.

Keywords: *Actinida deliciosa*, GDP-L-galactose phosphorylase (*GGP*) gene, Kiwi, T/A cloning, Vitamin C

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Introduction

Vitamin C is a common name of L-ascorbic acid (AsA) that derived from a six-carbon sugar (L-threo-hex-2-enono-1,4-lactone).[1] Vitamin C is a water-soluble antioxidant that has a predominantly protective role and acts as a cofactor in the synthesis of carnitine and collagen requirement of cardiovascular function, repair, and maintenance of cartilage, bone, and teeth. It also acts as an important component of the skin, scar tissue, tendons, ligaments and blood vessels, as well as the development of immune cells and iron absorption, and is necessary for healing. Human beings with other primates, bats, and guinea pigs aren't able to synthesize it, so they have to obtain through food sources.[2,3] As vitamin C is soluble in water, it would easily dispose of the body and is not stored. Therefore, it must be supplied by food sources.

Although we can obtain vitamin C from fresh meat, it degrades by warming the meat and usually vitamin C provided from plant sources. There is a high level of vitamin C in vegetables and fruits while usually seeds and dry seeds have a low level of vitamin C.[2,4]

The Smirnov-Wheeler pathway is one of the most important pathways for ASA biosynthesis in plants which consists of GDP-D-mannose and contains L-galactose arrangement.[5] GDP-L-galactose phosphorylase (*GGP*) enzyme is responsible for conversion of GDP-L-galactose to L-galactose-1-P in the first step of the Smirnov-Wheeler pathway in plants. This enzyme is coded by *GGP* and also called *VTC2*, knows as first regularly point in vitamin C pathway (Figure 1).

In this regard, over-expression of *GGP* in plants can significantly increase the concentration of ascorbate in the tissues.[6,7] In order to increase vitamin C, *GGP* gene was isolated from kiwi and transferred to the Arabidopsis plant As a result, increasing the expression of *GGP* gene increased the content of vitamin C in the Arabidopsis leaf up to

Figure 1. Vitamin C Biosynthesis Pathway. 1: Smirnov-Wheeler pathway, 2: L-glucose pathway, 3: Direction based on Myo-Inositol pathway, 4: D-glucuronic acid pathway.[8,13]

4-folds.[7] The purpose of this study was isolation, cloning and characterization of the gene encoding the *GGP* enzyme.

Materials and Methods

Experimental Reagents

T4 DNA ligase, Taq DNA polymerase, DNA restriction enzymes, One Step RT-PCR Master Mix, RNA extraction and DNA gel extraction kits were supplied from Ferments Company. The *Escherichia coli* DH5 <alpha> was used in all molecular biological experiments. Plasmid of pTG19 was used for cloning and sequencing.

RNA Isolation

Total RNA was isolated from *Actinida deliciosa* by RNA extraction Ribospin™ Plant kit. Its quality and quantity were analyzed by gel electrophoresis and spectrophotometer, respectively. Total extracted RNA was used as template for Reverse transcription polymerase chain reaction (RT-PCR) reaction to make cDNA. The cDNA was used as template for PCR reaction for isolate *GGP* gene.

Primer Design and PCR Amplification

The *GGP* gene of *A. deliciosa* amplified by PCR using EX Taq DNA polymerase and specific primers GGPF: 5' aga GGATCC actagt ATG ttg aag atc aag agg gtt c 3′ with a *Bam*HI and *Spe*I site at the 5′ end (underlined) and GGPR: 5′ cat GAGCTC cacgtg TCA gtg ctg aac tag gca t 3′ with *Sac*I and *Pml*I site at the 5′ end (underlined). These primers were synthesized based on the reported *GGP* gene sequences of kiwifruit available at NCBI (GenBank accession number: GU339036, KC146048.1, KC146049.1, EF379384.1).

Cloning and Sequencing

The PCR product was extracted by a DNA gel extraction kit (South Korea's GeneAll Company's DN kit) and ligated to pTG19 plasmid with T4 DNA ligase and then transferred to competent cells of *E. coli* by means of heat shock method. For transformation 200 µL of competent cells and 20 µl of recombinant vector (pTG19 + Insert) were placed on ice for 30 minutes, followed by a heat shock at 37°C for 5 minutes and placed back on ice for 2 minutes. Then 1ml of LB medium without antibiotic was added and incubated at 37°C for 2 hours with 180 rpm agitation. The bacterial solution was separated on a selectable solid LB medium containing 100 mg l^{-1} ampicillin and incubated at 37°C for 16 hours for selection of recombinant bacteria. Molecular analyses (such as; colony PCR, digestion with restriction enzyme and sequencing) were performed to verify the presence of the distinct insert. Plasmid was purified for sequencing using the standard method.[9] Sequencing was performed with M13F and T7 standard primers, using the capillary method with ABI system by Macro gene, Korea.

Ethical Considerations

This article does not contain any studies with human participants or animals performed by any of the authors.

Results

The *GGP* gene fragment (1383 bp) was obtained by PCR reaction via specific primers GGPF and GGPR (Figure 2). The *GGP* was cloned into the pTG19 plasmid. The positive colonies were confirmed by colony PCR, digestion with restriction enzymes (*Bam*HI and *Sac*I) and sequencing with M13F and T7 standard primers. Sequencing analysis of the *GGP* gene showed that this fragment contains 1383 bp and the start and stop codons were ATG and TGA respectively (Figure 3).

Our sequence was submitted to NCBI server (https://www.ncbi.nlm.nih.gov) for blasting. Various *GGP* sequences (18 accession numbers) were extracted from GenBank. Four

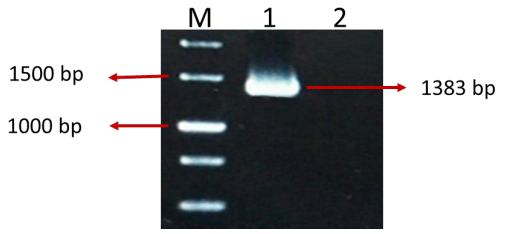

Figure 2. *GGP* Gene Amplification. M: DNA size Marker (1 Kb), PCR amplification of this gene is shown in lane 1 and Negative control in lane 2.

sequences belonged to *Actinidia* (GU339036, KC146048.1, KC146049.1 and EF379384.1). After studying the available sequences in the NCBI data bank, 18 sequences were chosen based on the complete encoding sequence (Table 1).

The alignment of our sequence was done with other *Actinidia* DNA sequences. The results based on Neighbor-Joining alignment showed that some of the mutations in nucleotides were related to the third nucleotide in a specific codon; in fact, with the nucleotide change, only codon usage changed and the amino acid remained unchanged. We see a change in the type of amino acid that is identified in yellow (Table 2).

Also, the nucleotide position number 745 that shown by R in NCBI bank had the high exchange rate of nucleotide.[6]

We used a web based translation tool (https://web.expasy.org/translate) for translating our sequence to protein sequence (Figure 3), then we aligned all 18 chosen protein sequences and our protein sequence by UPGMA method. The phylogenic tree showed that the 18 sequences were placed into six groups, our sequence and five of them (*Actinidia deliciosa*, *Actinidia eriantha*, *Actinidia rufa*, *Camellia sinensis*, *Actinidia chinensis*), were located into a group (group 2). Also, the minimum distance of sequences was observed between *GGP* CLON and *Actinidia chinensis*. The highest distance between *GGP* CLON, *Actinidia chinensis, Zea mays* was observed (Figure 4).

After studying the reported sequences for *GGP* gene in NCBI, 18 sequences were chosen (Table 1) and they were aligned by the MEGA7 software and based on UPGMA method with our sequence the phylogenic tree was reconstructed (Figure 4). The Phylogenic tree showed that 18 sequences were placed into ten groups, our sequence and five of them (*A. deliciosa*, *A. rufa*, *A. chinensis*, *A. eriantha* and *C. sinensis*) were located in the same group (group 1).

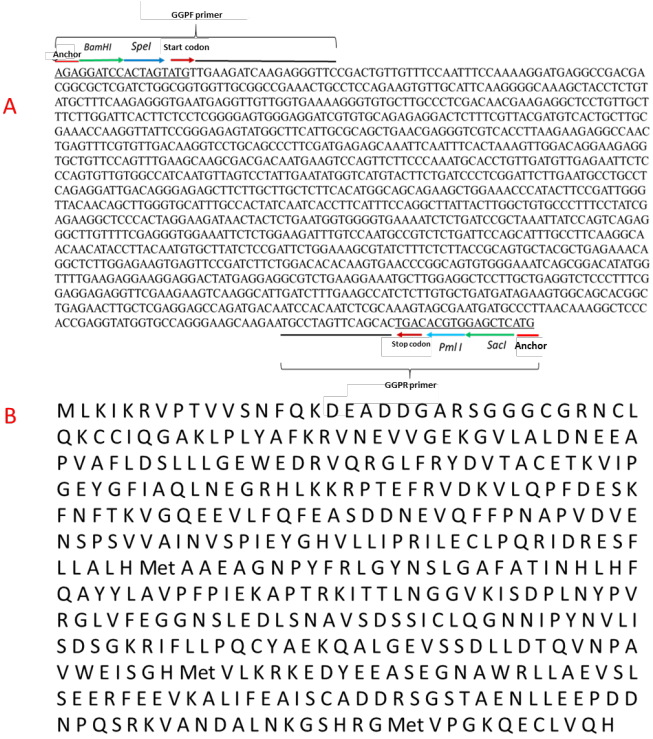

Figure 3. Sequence Analysis of *GGP* Gene and Predicted Protein Sequence. A: DNA sequence with primers site, start and stop codons and restriction enzyme sites. B: predicted protein sequence of *GGP* gene (https://web.expasy.org/translate).

The studies showed that phylogenic trees (for DNA and protein) have different groups. Also, *Zea mays*, *Zea mays(2)* and *Malus domestica* plants separately formed a group.

In the protein phylogenic tree, *Moringa oleifera* and *Malpighia glabra* were grouped in one group that indicates, they have more similarity in protein sequences, despite the difference in their DNA sequences.

Discussion

In this research, the *GGP* gene was isolated from the cDNA of *A. deliciosa* and subcloned into pTG19-T vector for sequencing. Result of sequencing showed the cDNA of *AcGGP* had a 1383 bp open reading frame (ORF). Whereas, Linster

Table 1. Information Sequences Used for Alignment

Plant	Gene bank	Pro ID	Plant	Gene bank	Pro ID
Our *GGP* Clone	-	-	*Arabidopsis thaliana*	AF508793.1	AAM34266.1
Actinidia deliciosa	GU339036.1	ADB85572.1	*Arabidopsis thaliana*	NM_124894.5	NP_200323.1
Actinidia rufa	KC146048.1	AGO32051.1	*Nicotiana tabacum*	NM_001324638.1	NP_001311567.1
Moringa oleifera	AB924665.1	BAP76192.1	*Nicotiana tabacum*	KJ808752.1	AII99836.1
Brassica rapa	JN157614.1	AET14214.1	*Actinidia eriantha*	KC146049.1	AGO32052.1
Camellia sinensis	KC505203.1	AGI78464.1	*Actinidia chinensis*	EF379384.1	ABP65665.1
Malpighia glabra	EU683446.1	ACG75920.1	*Solanum lycopersicum*	JQ517313.1	AFD54988.1
Malus domestica	NM_001293885.1	NP_001280814.1	*Zea mays*	EU966173.1	ACG38291.1
Solanum tuberosum	NM_001288371.1	NP_001275300.1	*Zea mays*	EU961505.1	ACG33623.1
Solanum tuberosum	NM_001288264.1	NP_001275193.1			

Table2 . Mutation Point in all *Actinidia* Species

Nucleotide Number	Clone GGP	*Actinidia deliciosa*	*Actinidia rufa*	*Actinidia eriantha*	*Actinidia chinensis*
33	T	T	T	T	C
60	C	A	C	C	G
75	T	T	T	T	C
129	G	G	A	G	G
159	T	G	T	T	A
181	G	T	G	G	T
190	C	G	C	C	G
196	A	G	A	A	G
220	T	T	C	T	T
228	A	G	G	A	A
234	C	T	T	C	T
276	T	C	C	C	C
312	G	A	A	G	A
337	C	C	C	C	T
387	C	C	T	T	C
423	T	T	T	C	T
473	A	A	A	T	A
492	C	C	C	T	C
507	T	T	T	T	G
586	C	T	C	C	C
587	G	C	G	G	G
608	A	G	A	A	A
669	A	G	G	G	G
708	C	A	C	C	C
723	T	T	T	T	A
744	G	G	A	A	A
745	G	R	G	G	G
747	T	C	T	T	T
756	T	C	T	T	T
765	G	G	A	G	G
770	C	T	C	C	C
819	T	G	T	G	T
821	C	T	T	T	T
865	T	A	T	T	T
888	C	A	C	C	C
889	G	G	A	G	G
916	G	G	G	A	G
961	C	C	C	C	T
963	T	G	T	C	T
972	C	T	C	C	C
978	G	A	G	A	G
984	C	T	C	T	C
987	C	C	C	T	C
999	G	G	G	A	G
1018	T	T	C	T	T
1020	C	T	C	C	C
1023	T	G	T	G	T
1099	G	G	C	C	G
1107	G	G	A	G	G
1164	C	C	T	C	C
1213	A	G	A	A	A
1227	G	A	G	G	G
1240	C	C	C	G	C
1255	G	G	G	A	G
1257	C	T	A	C	C
1258	A	A	A	G	A
1263	A	T	A	T	A
1271	G	G	A	G	G
1272	C	T	T	T	C
1273	A	G	G	G	A
1277	T	A	T	T	T
1279	G	G	A	G	G
1281	G	A	G	G	G
1301	G	G	G	C	G
1309	C	T	C	C	C
1311	A	T	A	A	A
1323	A	G	A	A	A
1326	G	A	G	G	G

Red font: effective mutation.

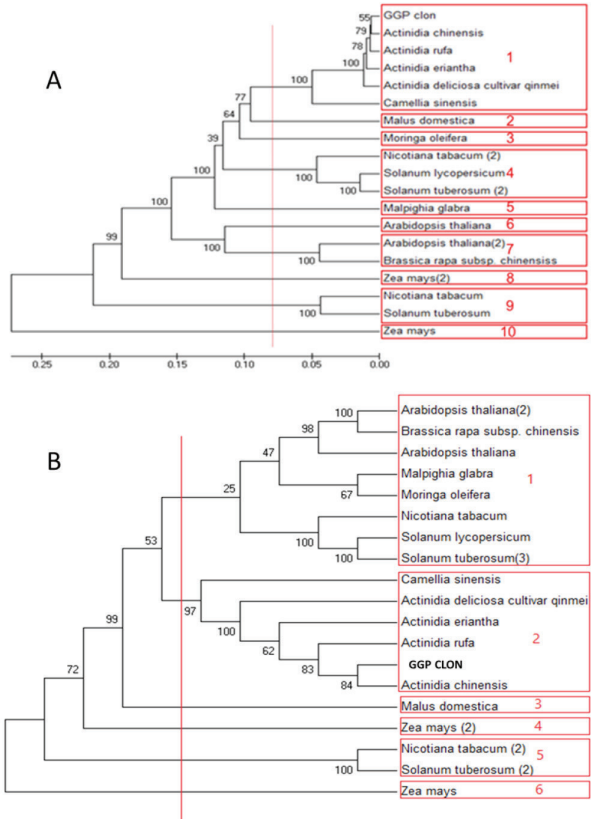

Figure 4. DNA and protein phylogenic tree, A: DNA tree with 10 groups, B: protein tree with 6 groups (by UPGMA method).

et al isolated *GGP* gene (VTC2 which encodes a GDP-L-galactose phosphorylase) from cDNA of *Arabidopsis thaliana* and then cloned in Champion pET100/D-Topo vector. They aligned *A. thaliana VTC2* sequence (amino acids) with *Oriza sativa*, *Homo sapines*, *Caenorhabditis elegans* and *Drosophila melanogaster*.[10] They found apparent orthologs in invertebrates, vertebrates and plants, including the *VTC2* gene product of *A. thaliana*.[10]

In a research, the leaves of the *M. oleifera* used for RNA extraction and cDNA synthesis for isolation of partial *GGP* gene with primers that was designed based on the conserved amino acid sequences of plant AsA biosynthesis enzymes. The isolated gene was subcloned into the pGEM-T Easy vector and sequenced. Then the gene-specific primers were designed and used for 5'- and 3'-rapid amplification of cDNA ends (RACE) to determine full-length cDNA sequences for *Moringa GGP*. The cDNA of *MoGGP* had a 1320 bp ORF that was predicted to encode a protein of 440 amino acids with a calculated molecular mass of 48963 Da.[11] Primary structure analysis using the Conserved Domain Database (CDD) suggested that *MoGGP* contains a histidine triad motif found in *A. thaliana GGP*.[11] Also they cloned and sequenced the *Moringa GGP* form genomic sequence.[11]

In a study, Urzica et al reported that the L-galactose pathway of ascorbate biosynthesis described in higher plants is conserved in green algae. They characterized recombinant *Chlamydomonas reinhardtii VTC2* as an active GDP-L-galactose phosphorylase. The *VTC2* cDNA was completely sequenced. It contains the entire predicted *VTC2* open reading frame, 1857 bp long, encoding a protein of 618 amino acids. BLASTp searches identified a *VTC2* homolog in *C. reinhardtii* exhibits 46% amino acid sequence identity to *A. thaliana VTC2*.[12]

Conclusions

Finally, according to our results, isolated *GGP* gene is a key enzyme in the biosynthetic pathway of L-ascorbic acid and it can be used to express in different plants for increasing vitamin C content.

Authors' Contributions

All authors contributed equally to this research.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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