Original Article

Isolation and Characterization of GDP-D-mannose 3, 5-epimerase (*GME*) Gene Impressive in Vitamin C Biosynthesis Pathway

Sayed Moein Zakari[1], Alireza Zebarjadi[2*]

Abstract

L-ascorbate acid is the scientific and common name for vitamin C. This vitamin is derived from L-threo-hex-2-enono-1,4-lactone. GME enzyme can modify GDP-D-mannose via epimerase effect and turns it to GDP-l-galactose. Thus, it creates interaction and relation between the synthetic pathway of vitamin C and the synthetic pathway of cell wall polysaccharides. Also, GEM enzyme produces GDP-l-glucose via another epimerase effect on GDP-l-galactose which is recognized as a new intermediate in vitamin C pathway of plants. In the biosynthesis pathway of vitamin C, GME has the most amount of protein protection. In this research, the GME gene of *Actinidia deliciosa* cultivar Hayward was cloned into the pTG19 plasmid. Sequencing analysis of the GME gene showed that this fragment contains 1161 bp. Results of blast showed that our sequence had high similarity (1973 score) with *Actinidia deliciosa* cultivar Qinmei and lowest similarity (1002 score) with Musa acuminate. According to the results of this study both phylogenic trees (DNA and protein) were divided into 7 separate groups. Also, *Chlamydomonas reinhardtii* and *Oryza sativa* Japonica in this dendrogram were placed in a separate group. Based on the results, *Vitis vinifera* was placed in two distinct groups in DNA and protein phylogeny trees. In contrast to DNA phylogenic tree in the protein phylogenic tree, all Solanums plants are grouped in one group that in dictate, although they are different in DNA sequencing, they are very similar in protein sequences.

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Introduction

The number of organic compounds is known as a vitamin that organisms are not able to synthesize it, and must be obtained from different food sources because these are essential compounds for the existence of organisms. L-ascorbate acid is the scientific and common name for vitamin C. This vitamin is derived from L-threo-hex-2-enono-1,4-lactone. In mammals, the last enzyme of the synthesize pathway of AsA is L-gulono-1,4-lactone oxidase, that the gene responsible for the synthesis of this protein in humans, primates and other mammals is strongly mutated and disabled. In the result, these organisms inevitably use external sources for obtaining vitamin C [1]. The global organization NAS (organization National Academy of Sciences) recommends daily using vitamin C for men and women 90 mg and 75 mg, respectively. One of the important vitamins in herbal food sources is vitamin C because humans obtain more than 90 percent of required vitamin C from vegetables and fruits [2]. This matter causes the most of researchers to look for developing of strategy that can increase the amount of vitamin C in plants. One of the important strategies in this regard is to increase the vitamin C production capacity and also to increase the ability of the plant to recycle its consumed vitamin C [3, 4].

Vitamin C synthesis pathway starts whit D-glucose in mammals. The synthesis pathway consists of turning D-glucose-1-P to UDP-D-glucuronic acid, UDP-D-glucuronic acid-1-P, D-glucuronic acid, L-gulonic acid and continues with gulono-1,4-lactone. In the next level glucone, 1-4 oxidase enzyme converts gulono-1, 4-lactone into the 2-keto-gulono-γ-lactone which itself automatically turns to L-ascorbic acid (Fig. 1) [5].

In plants unlike mammals that have only one pathway for the synthesis of vitamin C, at least 4 synthetic pathways are proposed. The Smirnoff-Wheeler pathway can be considered as the first biosynthetic pathway is discovered about vitamin C in plants that begins whit L-galactose sugar [6].

Molecular interfaces of guanosine diphosphate (GDP)-mannose and GDP-L-galactose can produce l-galactose sugar via mannose-1-phosphate, then L-galactose dehydrogenase-depended-NAD+ converts L-galactose sugar to L-galactose 4,1 lactone which this substance is oxidized by mitochondrial dehydrogenase and turns to L-Ascorbic acid [7, 8] (Fig. 1). GME enzyme can modify GDP-D-mannose via epimerase effect and turns it to GDP-l-galactose. Thus, it creates interaction and relation between the synthetic pathway of vitamin C and the synthetic pathway of cell wall polysaccharides. Also, GEM enzyme produces GDP-l-glucose via another epimerase effect on GDP-l- galactose which is recognized as a new intermediate in vitamin C pathway of plants. In the biosynthesis pathway of vitamin C, GME has the most amount of protein protection [9, 10]. Under a tensional condition, GME has a regulatory role in the synthesis of vitamin C and can provide a balance between vitamin C and the synthesis of cell wall monosaccharides. Besides, in the biosynthetic pathway of vitamin C, GME creates a control point that regulates this pathway in plants [9-11] (Fig. 2). Researchers have indicated that

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the content of vitamin C declines by suppressing *GME* gene via RNAi silencing in plants, which generates the leaves of the plants to become white and create evolutionary disagreements with ROSes aggregation.

However, the high expression of *GME* gene in transgenic plants that they are modified by this gene cusses severe changes in the mechanical properties of the cell wall, which makes it compact and these changes are visible in the plant itself and its fruits [12].

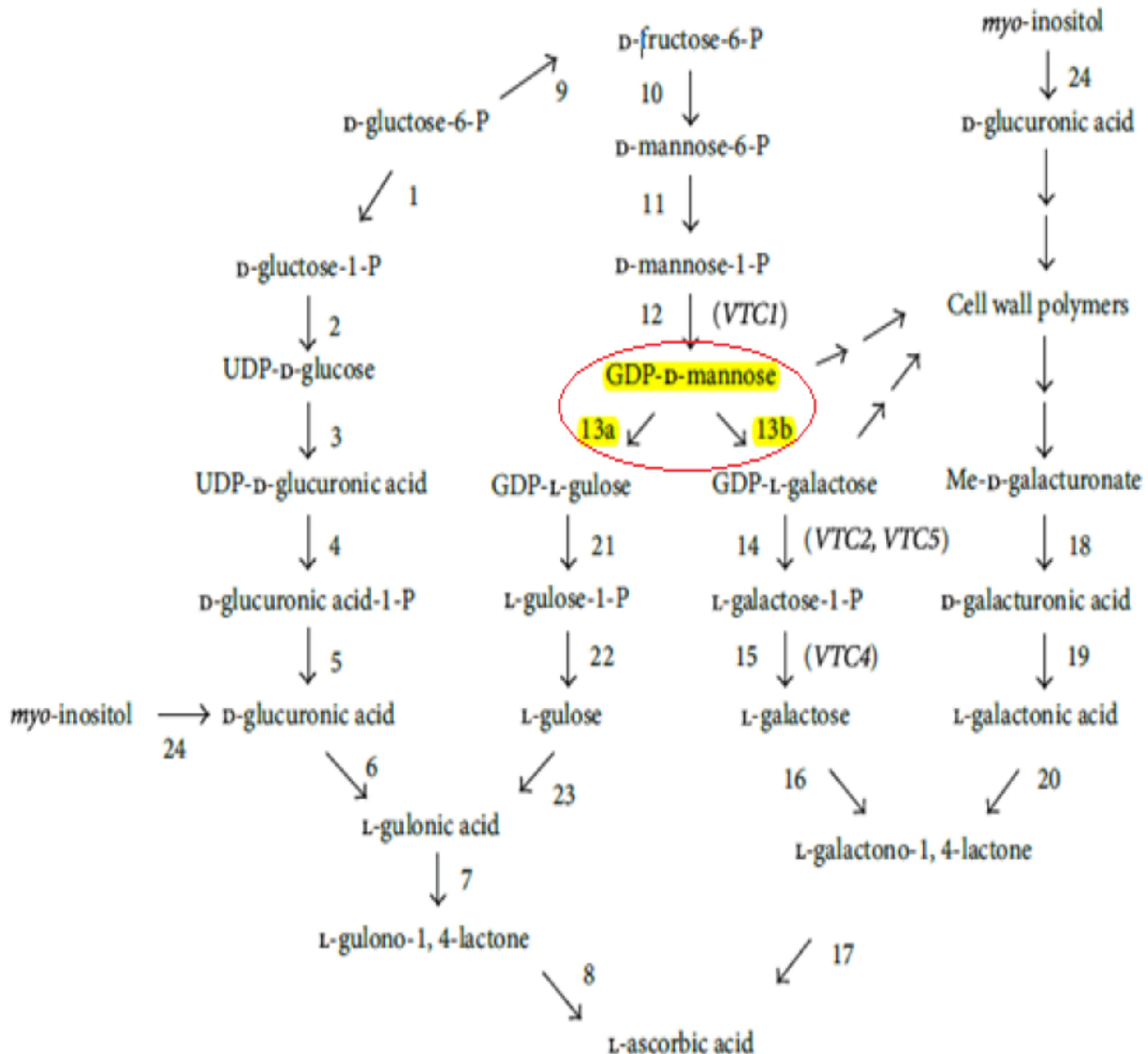

Figure 1. Vitamin C biosynthetic pathway in plants and animals [15].
Reactions 1–8 represent the pathway in animals and reactions9–24 represent the pathways in plants. Enzymes in each pathway are 1, phosphoglucomutase; 2, UDP-glucose pyrophosphorylase; 3, UDPglucose dehydrogenase; 4, glucuronate-1-phosphate uridylyltransferase; 5, glucuronate 1-kinase; 6, glucuronate reductase; 7, aldonolactonase (aka. gluconolactonase); 8, gulono-1,4-lactone oxidase or dehydrogenase; 9, glucose-6-phosphate isomerase; 10, mannose-6-phosphate isomerase; 11, phosphomannose mutase; 12, GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase) (VTC1); 13, GDP-mannose-3′, 5′-epimerase; 14, GDP-L-galactose phosphorylase (VTC2 and VTC5); 15, L-galactose-1-phosphate phosphatase (VTC4); 16, L-galactose dehydrogenase; 17, L-galactono-1,4-lactone dehydrogenase; 18, methylesterase; 19, D-galacturonate reductase; 20, aldonolactonase; 21, phosphodiesterase; 22, sugar phosphatase; 23, L-gulose dehydrogenase; 24, myo-inositol oxygenase. Adapted from Agius [15].

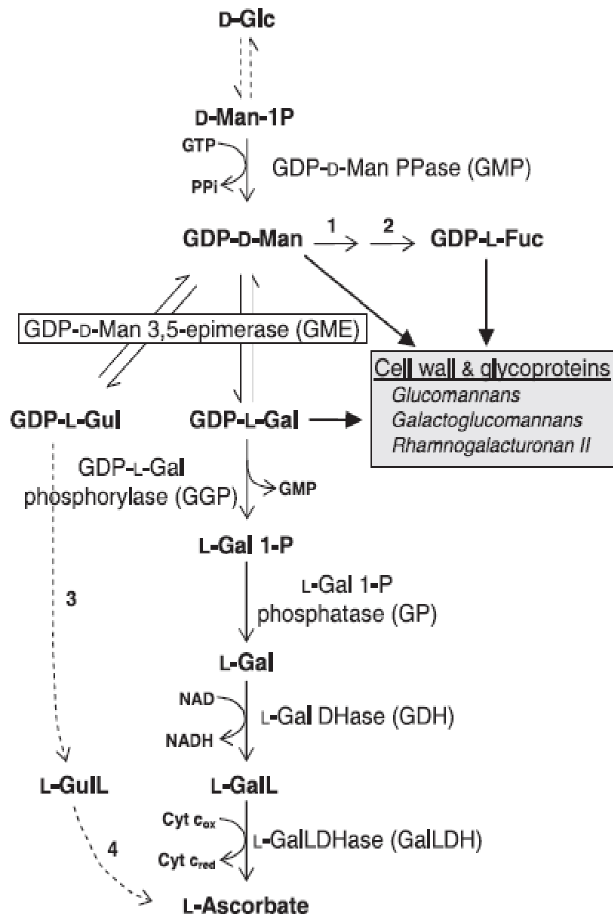

Figure 2. The control point of GME in vitamin C pathway. GME enzyme has three effective paths that one of them effect on cell wall and another effect on vitamin C [12].

Materials and Methods

Experimental Materials

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 and EX Taq DNA polymerase was supplied from GeneON Company, Germany.

RNA extraction and cDNA synthetics

Total RNA was isolated from *Actinidia deliciosa* Hayward by RNA extraction kit. Its purity and concentration were analyzed by gel electrophoresis and spectrophotometry. Total RNA was used as a template for RT-PCR reaction to make cDNA.

Primer design and PCR amplification

The *GME* gene of *Actinidia deliciosa* Hayward genome was amplified by PCR using EX Taq DNA polymerase and specific primers including GMEF: 5'AGA GGA TCC ACT AGT ATG GGA AGC ACC AGT GAA TC3′ with a *BamHI* and *SpeI* site at the 5′ end (underlined) and GMER: 5'CAT GAG CTC CAC GTG TCA TTC TTT GCC ATC AGC3' with *SacI* and *PmlI* site at the 5′ end (underlined). These primers were synthesized based on the reported *GME* gene sequences of kiwifruit available at NCBI (GenBank accession number: GU339037, JN132110.1).

Cloning and sequencing

The PCR product was extracted by a DNA gel extraction 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, 20 µl of the recombinant vector (pTG19 + Insert) was added in 200 µl competent cells and 20 µl of the recombinant vector (pTG19 + Insert) were placed in ice for 30 min, followed by a heat shock at 37°C for 5 min and placed in ice for 2 min. Then 1 ml of LB medium without antibiotic was added and the mixture was incubated at 37°C for 2 h with 180 rpm agitation. The bacterial solution was then poured on a selectable solid LB medium contains 50 mg/L ampicillin and incubated at 37°C for 16 h for selection of recombinant bacteria. Molecular analysis was performed to verify the presence of the distinct insert. Recombinant plasmid was purified for sequencing using the standard method (Sambrook, 2001). Sequencing was performed with M13F and T7 standard primers, using the capillary method with ABI system by Macrogene, Korea.

Results

The *GME* gene fragment (1161 bp) was isolated by PCR reaction via GMEF and GMER primers (Fig. 3).

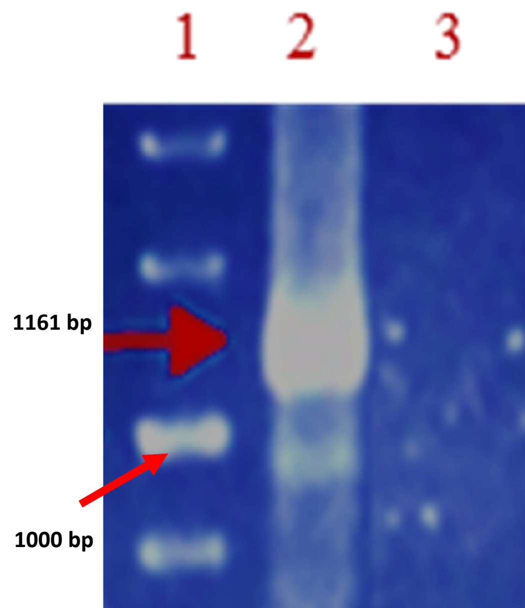

Figure 3. PCR product of *GME* gene. Lane 1: DNA size marker (1 Kb); Lane 2: PCR product; Lane 3: Negative control.

The *GME* gene was cloned into the pTG19 plasmid. The putative positive colonies were confirmed by PCR, restriction enzymes (*BamHI* and *SacI*) analysis and sequenced with M13F and T7 standard primers. Sequencing analysis of the *GME* gene showed that this fragment contains 1161 bp and the start codon and stop codon were ATG and TGA respectably (Fig. 4A).

Among the numerous sequences of the *GEM* gene in the NCBI database, 24 samples were selected (Table 1).

They were aligned by the MEGA7 program, based on Neighbor-Joining method with our sequence, and the phylogenic tree was draw (Fig. 5A). The phylogenic tree showed that 25 sequences were placed into seven groups. Our sequence and five of them including *Actinidia deliciosa*, *Actinidia rufa*, *Camellia sinensis* (GME1), *Camellia sinensis* (GME2), and *Vitis vinifera* were located into a group (group 1).

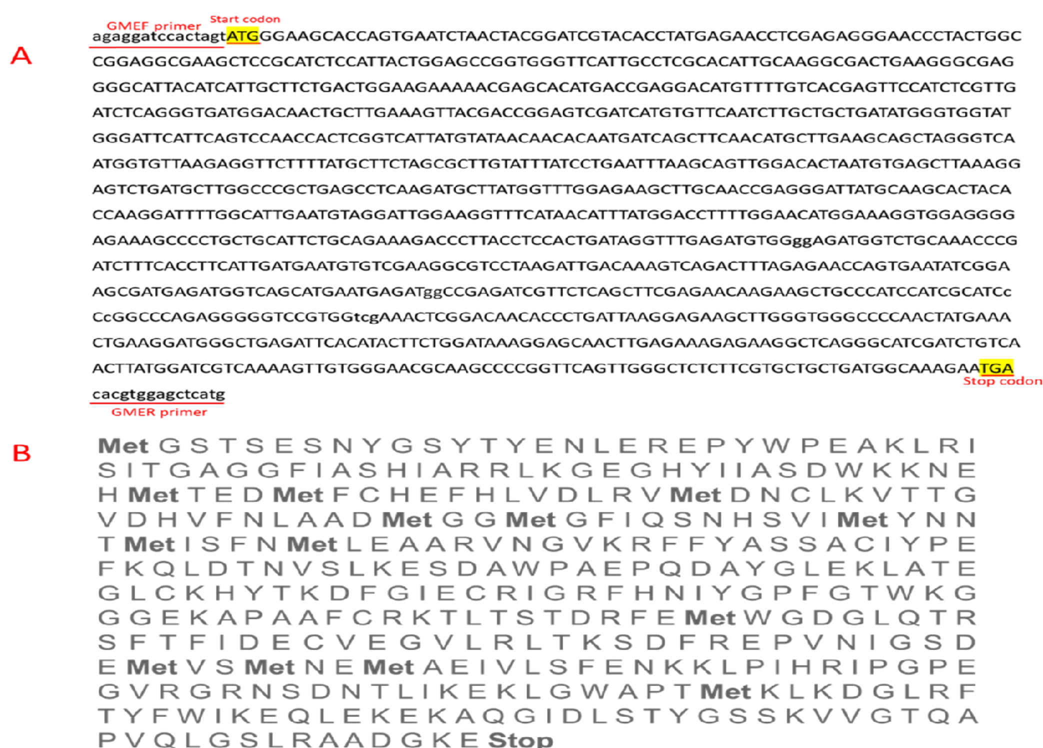

Figure 4. Sequence analysis of *GME* gene and predicted protein sequence. A: DNA sequence with its primer site, start and stop codon. B: predicted protein sequence of *GME* gene by https://web.expasy.org/translate.

Table 1. Information of 25 sequences including our GME clone and 24 other sequences which used for alignment.

	Name	GenBank	Pro ID	NO.	Name	GenBank	Pro ID
1	GME CLONE	-	-	15	*Chlamydomona reinhardtii*	XM_001689445.1	XP_001689497.1
2	*Actinidia rufa*	JN132110.1	AEK22069.1	16	*Malpighia glabra*	DQ229167.1	ABB53472.1
3	*Actinidia deliciosa cultivar qinmei*	GU339037.1	ADB85573.1	17	*Glycine max*	FJ619111.1	ACU51229.1
4	*Solanum pennellii*	NM_001323467.1	NP_001310396.1	18	*Oryza sativa Japonica Group*	AB193582.1	BAD66930.1
5	*Solanumlycopersicum (GME2)*	NM_001247660.2	NP_001234589.1	19	*Citrus unshiu putative*	HQ224947.1	ADV59924.1
6	*Solanumlycopersicum (GME2.1)*	GQ150165.1	ACS45394.1	20	*Prunuspersica*	AB457582.1	BAH03299.1
7	*Solanumlycopersicum (GME1)*	NM_001247805.2	NP_001234734.1	21	*Brassica rapa subsp*	JN157613.1	AET14213.1
8	*Arabidopsis lyrata subsp. lyrata*	XM_002874405.1	XP_002874451.1	22	*Rosa roxburghii*	KC782575.1	AGO64481.1
9	*Solanum-melongena*	KU356957.1	AOW42606.1	23	*Medicago sativa*	KF935649.1	AHK12705.1
10	*Vitis vinifera*	NM_001281039.1	NP_001267968.1	24	*Prunus avium*	KX196287.1	APO15253.1
11	*Daucus carota*	KY347804.1	AQM57026.1	25	*Arabidopsis thaliana*	NM_001203488.1	NP_001190417.1
12	*Caragana korshinskii*	FJ603689.1	ACM47361.1				
13	*Camellia sinensis (GME2)*	JX624165.1	AGQ44774.1				
14	*Camellia sinensis (GME1)*	KC477767.1	AGI78462.1				

Our sequence was submitted to NCBI server (www.ncbi.nlm.nih.gov) for blasting. The result of BLAST showed various *GME* sequences (25 accession numbers) that had similarity with our sequence. Two sequences of these belonged to *Actinidia* (JN132110.1, GU339037.1). After studying on the results of our blasted sequence in the NCBI data bank between similar sequences, 24 sequences were chosen based on the complete encoding sequence and protein sequence that included crops, ornamental, wild species plants and single green algae called *Chlamydomonas reinhardtii* (Table 2). Results of blast showed that our sequence has high similarity (1973 score) with *Actinidia deliciosa* cultivar Qinmei and lowest similarity (1002 score) with Musa acuminate. The multiple alignments were down by MEGA7 software.

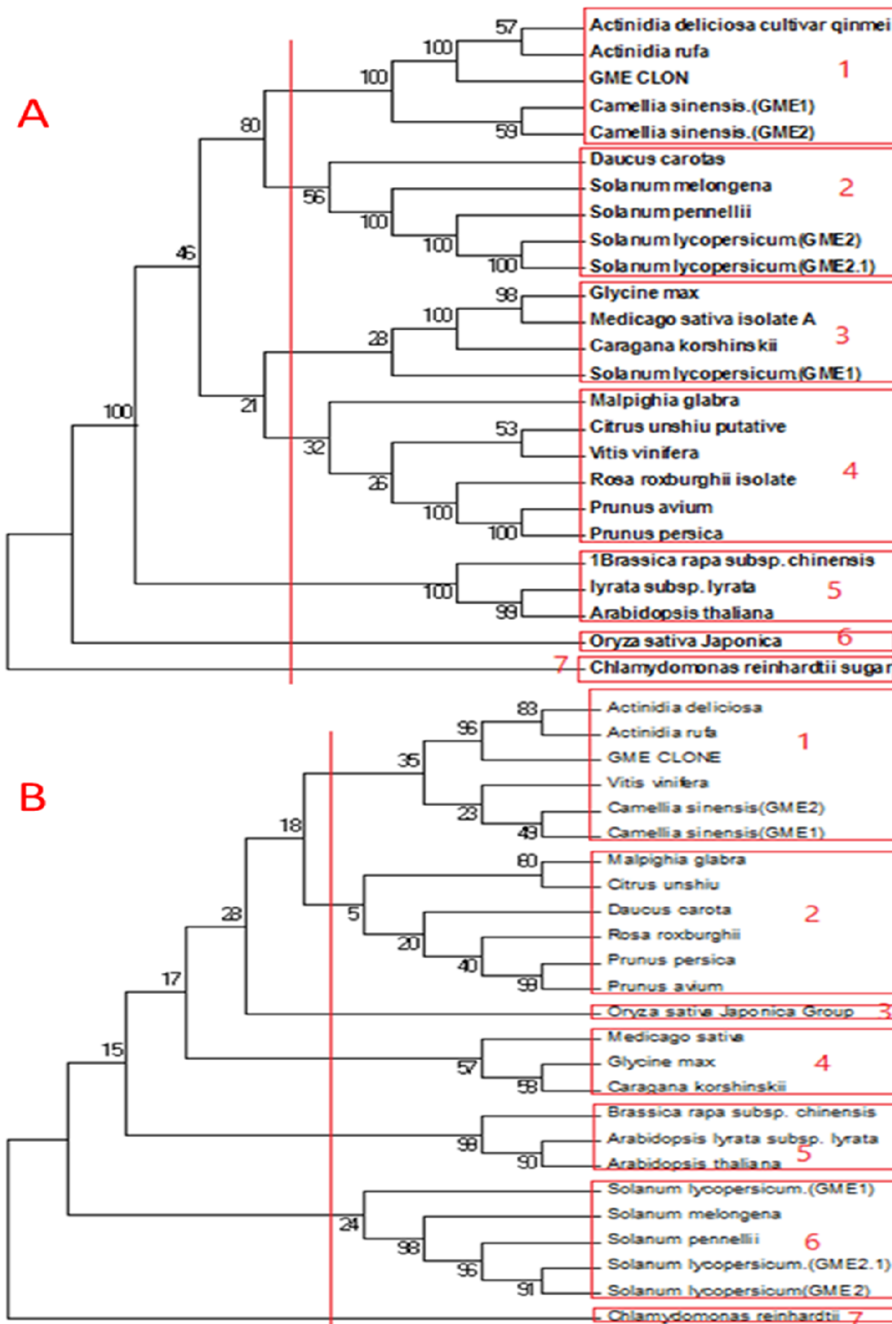

Figure 5. Phylogenetic trees of GME sequences, which is drawn based on multiple alignments for the 25 sequences studied. A: DNA phylogenetic tree according to which the plants are divided into seven groups. B: protein phylogenetic tree that was grouped into 7 parts.

We did first alignment between our sequence (GME clone) and other *Actinidia* DNA sequences based on Neighbor Joining method. Results showed the most of the mutations in nucleotides are 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. This is called the Wobble phenomenon and leads to ineffective mutations (Table 2).

We also used expasy site (https://web.expasy.org/translate) for translating our sequence to protein (Fig. 4B), then we aligned all 24 chosen protein sequences with our protein sequence by UPGMA method. The phylogenetic tree was drawn based on the alignment of our sequence and 24 GME protein sequences by UPGMA method. (Fig. 5B). The phylogenetic tree showed that the 25 sequences were placed into seven groups, our sequence and five of them including *Actinidia deliciosa*, *Actinidia rufa*, *Camellia sinensis* (GME1), *Camellia sinensis* (GME2), and *Vitis vinifera* were located into a group (Group 1).

Also, the minimum distance of sequences was observed between *Solanum lycopersicum* (GME2) and *Solanum lycopersicum* (GME2.1). The highest distance between *Solanum lycopersicum* (GME2), *Solanum lycopersicum* (GME2.1) and *Chlamydomonas reinhardti* sequences was observed.

The results showed that both phylogenic trees were divided into 7 separate groups. Also, *Chlamydomonas reinhardtii* and *Oryza sativa* Japonica were placed in separately group. Also in DNA phylogenic tree (Fig. 5A), *Vitis vinifera* was placed in a separate group in comparison with protein phylogenic tree (Fig. 5B). In contrast to DNA phylogenic tree, in the protein phylogenic tree, all Solanums plants were grouped in one group that indicate, although they are different in DNA sequencing, they are very similar in protein sequences. The results of this study showed that *Vitis vinifera* and our sequence were placed into the group 1 in protein phylogenic tree, but *Vitis vinifera* with *Prunus avium*, *Rosa roxburghii*, *Citrus unshiu putative*, *Malpighia glabra*, and *Prunus persica* were placed into the group 4 in DNA phylogenic tree. However, the remaining plants listed were placed into the same group in both phylogenic trees.

Studies on sequences showed that some of the mutations in the nucleotide sequences have been altered in their codons; in fact, the made mutation was effective, these mutations are highlighted (Table 2). The results showed that in position 545, the amino acid glutamic acid (acidic, polar, with a negative charge and hydrophobicity of -3.5) was converted to glycine (aliphatic, nonpolar with anhydrous -0.4) by changing of nucleotide A to G in our sequence

Table 2. The results of alignment between our sequence and other *Actinidia* DNA sequences.

Our sequence		***Actinidia_deliciosa***		***Actinidia_rufa***	
Position	Nucleotide	Position	Nucleotide	Position	Nucleotide
48	C	48	T	48	C
99	T	99	T	99	C
147	C	147	C	147	A
237	C	237	T	237	C
264	T	264	C	264	C
267	G	267	G	267	T
345	T	345	T	345	A
411	G	411	G	411	A
456	G	456	A	456	G
477	A	477	G	477	G
525	G	525	A	525	A
545	G	545	A	545	A
570	T	570	C	570	C
597	G	597	A	597	G
624	A	624	C	624	C
640	G	640	A	640	A
711	T	711	C	711	C
759	C	759	T	759	T
774	A	774	G	774	G
786	T	786	C	786	C
810	C	810	T	810	T
884	G	884	A	884	A
888	C	888	T	888	T
891	C	891	A	891	T
903	G	903	C	903	G
912	T	912	A	912	T
976	G	976	G	976	A
1005	A	1005	A	1005	C
1050	A	1050	A	1050	G
1059	A	1059	A	1059	G
1062	G	1062	G	1062	A

And in position 640, the amino acid arginine (basic, basic polar, positive, hydropathy of -4.5) was converted to glycine by changing of nucleotide A to G in our sequence. In position 884, the amino acid histidine (basic aromatic, basic polar, positive (10%), neutral (90%), hydropathy of -3.2) was converted to arginine by changing of nucleotide A to G in our sequence. In position 976, the amino acid aspartic acid (acidic, acidic polar, negative, hydropathy of -3.5) was converted to asparagine (amide, polar, neutral, hydropathy of -3.5) by changing of nucleotide G to A in *Actinidia rufa*.

Discussion

In this research, based on the sequences in the NCBI database, two special primers were designed and they were used to isolate the *GME* gene from the cDNA source of *Actinidia deliciosa* cultivar Hayward. In current study, the *GME* was isolated from *Oryza sativa* and was blasted with *Arabidopsis thaliana* GME. Results showed 80% similarity between two sequences [13].

Also, in a research, leaves, stems and roots of *Arabidopsis* and *Alfalfa* were used for extraction of RNA and cDNA synthesis for isolation of *GME* gene [14]. The most similarity (1973 score) was obtained with kiwi *Actinidia deliciosa* cultivar Qinmei with access number (GU339037.1) and the lowest similarity (1002 score) was obtained with species of *Banana* strain called *Musa acuminata* with access number (XM_009394784.2). In this research, based on the protein sequences and phylogeny tree, the samples were divided into seven distinct groups, the protein sequence of isolated gene of *Actinidia deliciosa*, *Actinidia rufa*, *Camellia sinensis* (GME1), *Camellia sinensis* (GME2), and *Vitis vinifera* were placed in one group. Also, the minimum distance between *Solanum lycopersicum* (GME2) and *Solanum lycopersicum* (GME2.1) was observed and the highest distance between the two sequences and *Chlamydomonas reinhardtii* was observed. Also *Chlamydomonas reinhardtii* and *Oryza sativa* each formed a group alone.

However, Watanabe *et al*., analyzed GEM sequences by ClustalW software. They conclude that isolated OsGME from rice has a 91% similarity with *Arabidopsis thaliana* and OsGME sequences have the most similarity with GMEs of *Zea mays* (97%), Sorghum bicolor (97%), *Hordeum vulgare* (93%), *Triticum aestivum* (92%), Lycopersicon esculentum (92% and 91%), *Medicago truncatula* (91%), *Lotus corniculatus* varjaponicus (98%), *Solanum tuberosum* (91%), *Mesembryanthemum crystallinum* (90%), and *Glycine max* (90%) [13].

Conclusion

We successfully isolated and cloned *GME* gene from Actinidia deliciosa cultivar Hayward into pTG19 plasmid. Finally, based on restriction sites that designed in primers we will sub cloning it in desirable plant binary vector for future researches.

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