Journal homepage www.jzs.univsul.edu.iq
Journal of ZankoySulaimani
Part-A- (Pure and Applied Sciences)

The occurrence of L-GALACTONO-1, 4-LACTONE DEHYDROGENASE (L-GalLDH) in L-ascorbate biosynthesis pathway from photosynthetic eukaryotes

Sevan O. Majed[1]

1Biology Department, College of Education, Salahaddin University-Erbil, Kurdistan-Iraq
sevan.majed@su.edu.krd

Article info

Original: 3 October 2015
Revised: 6 February 2016
Accepted:
25 February 2016
Published online:
20 September 2016

Key Words:
Ascorbate, L-Galactono-1,4-lactone dehydrogenas, phylogenetic analysis, gene duplication events.

Abstract

L-Galactono-1, 4-lactone dehydrogenase (L-GalLDH) is a key enzyme that catalyzes the last step in the L-ascorbate pathway in biosynthetic eukaryotes. It is widely distributed in the different lineages of photosynthetic eukaryotes. Here, we hypothesized that there is a close evolutionary relationship between L-GalLDHsequences of these eukaryotic lineages. To test this hypothesis, we collected all homologous L-GalLDHsequences of photosynthetic eukaryotes from the NCBI databases. This article reports two results. Firstly, phylogenetic L-GalLDHtree shows that different L-GalLDHs in different species have an evolutionary relationship with each other because these enzymes are descended from a common ancestral gene via gene duplication events at the earliest stage of evolution. The gene duplication events can lead to an emergence of multiple paralogous copy within the species called as In-paralogs and also in different species called as out-paralogs. Moreover, the speciation events can lead to the formation of different L-GalLDHproteins in different species. Because they share from a common ancestral gene, theyhave a close sequence similarity and functional similarity with each other. Secondly, biochemical experimental evidence reveals the presence and absence of the activity of the L-GalLDHenzyme in some lineages of photosynthetic eukaryotes; plants, chlorophyta, phaeophyceae, bryophyta, and pteridophyta. It is worth highlighting a fact that L-GalLDHprotein was only present in the species of a vascular plant (*Pisumsativum*), chlorophyta (*Blindingia minima*) and rhodophyta (*Porphyra purpurea*). This research study attempted to provide a further insight into the L-GalLDHenzymes have been functionally and structurally evolved over distinct lineages of the eukaryotes and over times.

Introduction

L-Galactono-1, 4-Lactone Dehydrogenase (EC:1.3.2.3,L-GalLDH, GLDHase and GLDase)is a key enzyme that involved in the last steps of L-ascorbate pathway for L-ascorbate biosynthesis in photosynthetic eukaryotes. L-ascorbate (AA) which is known as ascorbic acid and vitamin c is a pivotal antioxidant and an enzyme cofactor [1]. Green plants and higher animals (but not humans) are able to produce AA.The content of AA is discovered in nearly all cell compartments; including the Mitochondria, Cytosols, Chloroplaststroma, Peroxisomes, Vacuoles and Apoplasts [2]. The AA level is not similar in different species and even compartments of the same species. This leads to the reason why some fruits contain an exceptionally high concentration[3].The process of AA production takes several steps. The terminal step in the pathway of L-ascorbate formation is the oxidation of L-Galactono-1,4-lactone to AA [1] and [2]. This

reaction is regularly catalyzed by L-GalLDH enzyme and FAD-linked enzyme of the Vanilly l- Alcohol Oxidase (VAO) flavor protein family that uses c -type cytochrome (*cytc*) as its electron acceptor. The activity of this enzyme is very important to regulate of the L-ascorbate pathway because photosynthetic eukaryotes cannot produce AA if this enzyme is not present [1]and [3]. Previous research studies had revealed that the location of L-GalLDH is in Mitochondria in association with respiratory complex I[3]**.**The activity of this enzyme is widely found in flowering plant species but the informationis hardly found in the groups of non-flowering plants like moss (Bryophyte), ferns (Skipemoss) and eukaryotic algae [2] and [4]**.**It has been observed that some species of the non-flowering plants are able to generate a low concentration of AA because of having low content of this enzyme and also the occurrence of L-isomer in eukaryotic algae has been confirmed by the ascorbic oxidase (AO) assay [5] and [6]**.**There is evidence that non-flowering plants contain a low concentration of the L-GalLDH enzyme and produce ascorbate via the oxidation of the Mannose/L-Galactose pathway, due to the nonappearance of label inversion and competent conversion of L-[^{14}C] Galactose and D-[^{14}C] Mannose to AA [7], [8] and [9]**.** In addition, a few number of research studies on other photosynthetic Protists proposed that Chrysophyta, Euglenophyta and Bacillariophyta may not be able to produce ascorbate through the Mannose/L-galactose pathway as hexose precursor is inverted from radiolabelling researches which is not expected by the Mannose/L-galactose pathway [10], [11] and [12]**.** In all photosynthetic organisms that synthesize AA, the last step is the oxidation of an aldono-1,4-lactone. As explained above, the L-GalLDH of land plants is a dehydrogenase enzyme and, where substrate specificity has been determined, this enzyme is very specific for L-galactose [13] and [14]**.** Unlike photosynthetic plants, animals and protists utilize L-gulonolactone (L-GulL) as their ascorbate precursor. In addition, fungi possess a C5 analogue, D-erythroascorbate, generated from D-arabinonolactone [15], [16] and [17]**.** Here, phylogenetic analysis will be performed to show the evolutionary relationship of this enzyme among vascular and nonvascular plants. Furthermore, biochemical experimentwill beused to investigate the presence or absence of L-GalLDH activity from the species of five groups: including Vascular plant, Bryophyta, Petridophyta, Rhodophyat, Chlorophyta. These species have not been tested, yet.

Material and Method

Assemble and align protein databases

In order to estimate sequence similarities and evolutionary relationships between different L-GalLDHsin vascular and nonvascular plants, phylogenetic tree will be constructed. First of all, Because the sequences of L-GalLDHof *Arabidopsis thaliana* were already available in the genomic sequences of National Center for Biotechnology Information(NCBIweb site), These L-GalLDH sequences were used as Blast query sequences to search for the available database (original dataset). After that, the complete homologous sequences (Identity= 50% to 100%), which is available in the publicly original database, were downloaded.

Phylogenetic tree of similar L-GalDHseuquences in photosynthetic eukaryotes

Phylogenetic tree for L-GalDH sequences was performed by means of Molecular Evolutionary Genetics Analysis version five (MEGA6.06). The MEGA6, which is accurate and sensitive method, can provide multiple sequence alignment (MSA) algorithm and maximum likelihood (ML) method. We used the MUSCLE alignment algorithm to align the collecting homologous sequences of L-GalDH (Identity= 50% to 100%) because the accuracy and speed of MUSCLE tool is extremely higher than ClustalW and also it takes a less time than ClustalW when aligning larger numbers of sequences [18] and [19]**.** The phylogenetic tree was then constructed.

The presence of L-GalLDH activityin vascular and non-vascular plants may be related to their evolutions

Plastids are major double-membrane organelle. They are existent in the different groups of all the vascular and some non-vascular plants. By the existence of plastids originated from primary endosymbiosis, the members of these groups have been recently classified [20]**.** The plastids are possibly increasing because

of the photosynthetic eukaryotes achieved the capability to change sun light into chemical energy, between 1-1.5 billion years ago, via endosymbiosis with a single Cyanophyta [21]. In recent years, seaweeds based on plastids have been classified into three major groups: red algae (have Rhodoplasts), green algae (have chloroplasts) and brown algae (Phaeophyceae) [22]. However, there were disagreements of the classification of these three groups, between *Protista* and *Plantae* [23]. As more research studies conducted, there have been several proofs and information that vascular plants, mosses, ferns and eukaryotic algae have been as sister clades. These evidences were the high similarities in DNA, gene and protein sequences between algal chloroplast and mitochondrial genomes and those of land plants, provided by a multi-genetic investigation of a fusion of thirteen nuclear markers [24], [25] *and* [26]. We hypothesized that the ascorbate pathways of different lineages of photosynthetic eukaryotes have a very close evolutionary relationship with each other. Phylogenetic tree is constructed to elucidate this relationship. It shows that the ascorbate biosynthesis pathway of mosses and ferns is more similar to one another than either is to vascular plants. The vascular plants diverged from them and that the differences between them happened more recently (*Figure: 1*). It can be also seen that the pathways of green, red and brown algae have a very close similarity with each other. The differences between red and brown algae happened more recently but the variation in green algae occurred more rapidly. There is evidence that the ascorbate biosynthesis pathway of red algae has been recently discovered and it is related to green algae. It can possibly be suggested that this pathway could be similar to those in green plants [25] and [27].More recent genetic studies have shown that the eukaryotic algae are more distantly related to other vascular plants, having radiated evolutionarily at the base of the vascular plant clade.

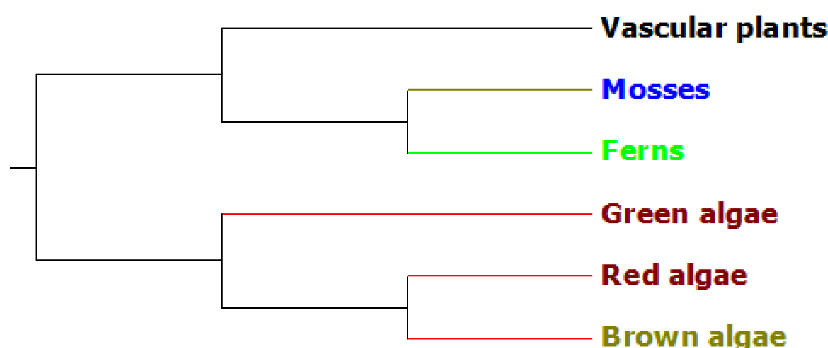

Figure-1:Phylogenetic tree of ascorbate biosynthesis pathway. The pathways in the lineages of photosynthetic plants have the evolutionary relationship with each other. It appears that plants, mosses and ferns are more similar than to one anther than either is to eukaryotic algae because they are descendent from a common ancestor and grouped in a clade.

The occurrence of L-Gaildhactivityin ascorbate pathway from photosynthetic eukaryotes may be related to their evolutions.

L-GalLDH (EC:1.3.2.3) enzyme belongs to the family of FAD_binding_4(PF01565). This family includes different enzymes that utilize FAD as a cofactor. Most of the enzymes are similar to oxygen oxidoreductase. According a research study conducted by Smirnoff and his colleagues in 2001, in vascular and non-vascular plants, different genes responsible for encoding the mitochondrial GalLDH are evolved [19]. In recent time, these different genes in the Mitochondria of most land plants and some seaweed have been sequentially investigated and analyzed. It is hypothesized that these L-GalLDH proteins in various species are sequentially and structurally similar and have an evolutionary relationship with each other. Phylogenetic tree based on comparison of homologous sequences for this enzyme from a variety of photosynthetic eukaryotes was constructed to show sequence similarity and evolutionary relationship. It showed that different L-GalLDHs in different species are descendent from a single common ancestor via gene duplication events at the early stage of evolution (*Figure: 2*). They are combined into one big tree. It

appears that the different genes in different species encode similar L-GalLDH because these different genes are evolved from a common ancestral gene via gene duplication events. The different L-GalLDHs have similar domains; therefore, they have similar functions in the intrinsic pathway of L-galactose. It can be seen that the tree was diverged into two major groups; group I and group II. The group I includes flowering and flowerless plants. It is vertically diverged into the clades of monocots and eudicots, mosses, ferns and eukaryotic algae for some reason of variation has occurred. The L-GalLDH enzymes in monocots have a very close sequence similarity to eudicots; therefore, they are combined together into two sister groups. However, the L-GalLDH sequences in ferns and mosses are more similar to one another than either is to eukaryotic algae because the fewest residual changes are happened. It can be detected that there are two different types of homology between different genes .Firstly, the paralogs include two types; out-paralogs and In-paralogs. The out-paraologs are paralogs that were duplicated before the speciation events but they are not necessarily in the same species (*figure: 2*). These are descended in the common ancestor of the pair of species. These proteins may have arranged in separate species through the loss of the reciprocal partner. In the figure shown, protein pairs *Arabidopsis thaliana* [AAO64860.1]-*Arabidopsis lyrata* [EFH52130.1] and *Capsella rubella* [EOA23228.1]-*Eutremasalsugineum* [ESQ45768.1] are out paralogs (in separate species, and descended from a more ancient shared duplication event). The in-paralogs in the same species may be evolved from a common ancestor via duplication events. For example, *Arabidopsis thaliana* contain four similar enzymes (Accession number: [AEE78347.1], [BAA95212.1], [AEE78348.1] and [AAO64860.1]) descended from a single ancestral gene (*Figure: 2*). The L-GalLDH is encoded by one gene (At3g47930) in *Arabidopsis*[9]**.** As shown in figure 3a, the sequence of [AEE78347.1] and [BAA95212.1] show the fewest residual changes and highest sequence similarity (identity=99%), so they are combined together in a clade. In addition, the fewest residual differences are shown between sequences of [AEE78348.1] and [AAO64860.1]: therefore, these are combined with previous sequences. Secondly, some species contain only one protein called as orthologs. The orthologs from different species are produced via speciation events. In the figure demonstrated, *Chlorella variabilis* [EFN51214.1] and *Coccomyxasubellipsoidea* C-169 [EIE19735.1] have high residual variations (*Figure: 2*). The identity value is 55%. They have the lowest similarity in the middle of the sequences (*Figure: 3b*). However, Group II which diverged from a common ancestor of Group I and that the difference between vascular and non-vascular plants occurred more recently, but undergone more raped variation since. It appears that the L-GalLDH sequences in the chlorophyta and Haptophyta species are more similar to one another than either is to rhodophyta species because the Rhdophyta may be evolved from a common ancestor of chlorophyta via speciation events (*figure: 2*).

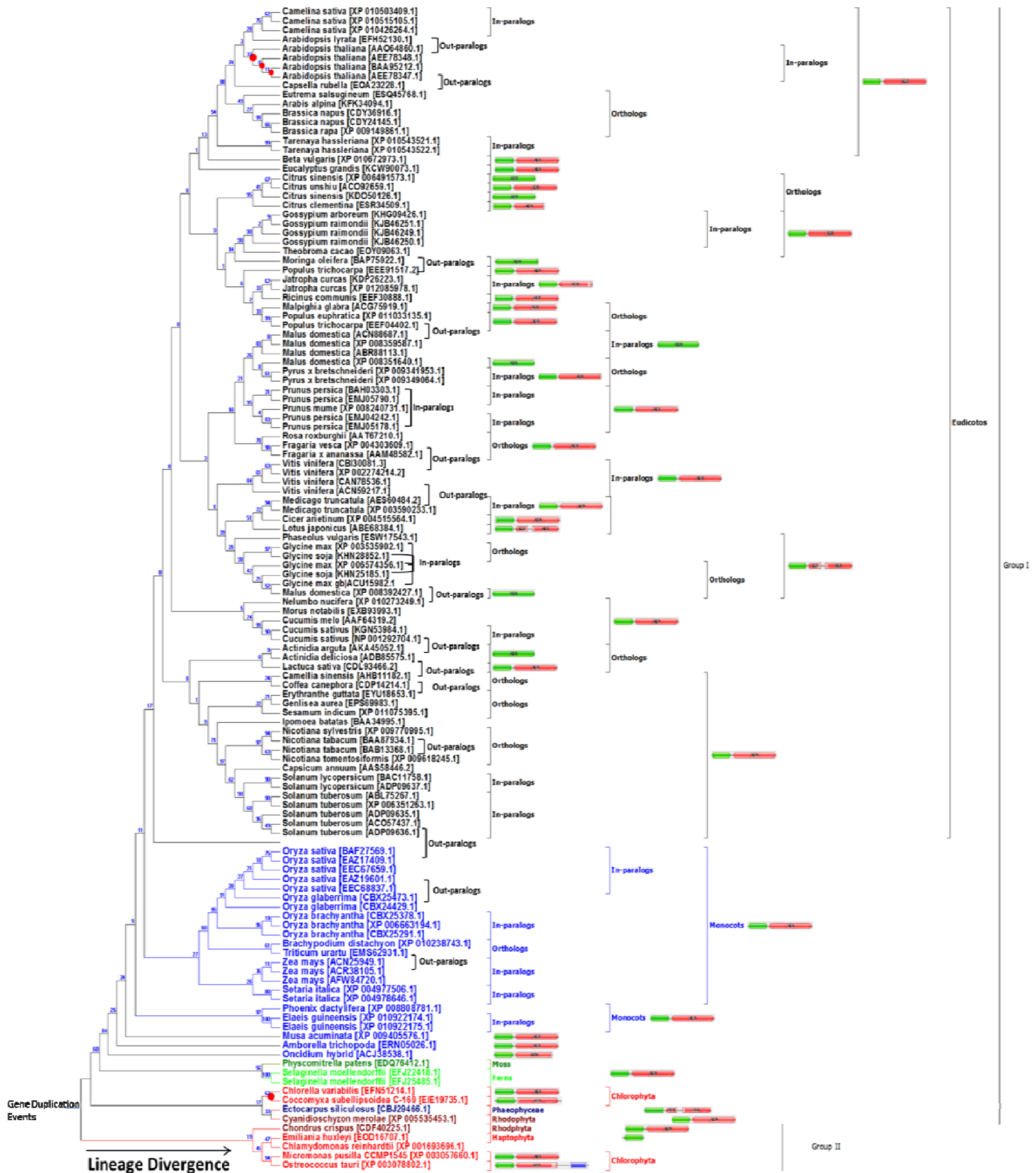

Figure-2**:**Phylogenetic tree of eukaryotic L-Gaildh sequences. Phylogenetic relationships between eukaryotic plants based on amino acid deduced sequences for representative l-Gaildh sequences. The sequences were aligned using Muscle of MEGA5 software. Phylogenetic tree was constructed with the maximum likelihood method, using the MEGA software, Version 6.06 [25]. Although, gene speciation can lead to the formation of orthologous L-Gaildhin different species, gene duplication events can lead to the formation of multiple paraologous copies in a species (In-paralogs duplicated after speciation event) and in different species (Out-paralogs duplicated before speciation event).The numbers on the branches represent bootstrap values used to estimate the reliability of phylogenetic tree. Bootstrap analyses were conducted on the basis of 1000 re-samplings of the sequence alignment. Each branch is annotated with the PFAM domain architecture of the source sequence**.** All sequences are labelled with protein Accession numbers. Sequences used in the alignment are available at (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Figure-3:a) Alignment of two in-paralogous sequences of 1) *Arabidopsis thaliana* gb|AEE783447.1 and 2) Arabidopsis thaliana dbj|BAA95212.1, with the corresponding dotplot. Note in both the sequence alignment and dotplot, the similarity value is 99%. **b)** Alignment of two Orthologous sequences of each *Chlorella Variabilis*gb|EFN51214.1 and *Coccomyxasubellipsoidea* c-169 gb|EIE19735.1, with the corresponding dotplot. Note in both sequence alignment and dotplot, the high similarity at the beginning and end of the sequences.

L-GalLDH role in L-ascorbate biosynthesis in green plants

The enzyme of L-GalLDHin L-ascoebatepathway of green plants has played an essential role in increase and reduction of L-ascorbate level. The enzyme activity and transcript level of L-GalLDH have been detected to significantly correlate with L-ascorbate rate in different plant compartments; though, there is

plant-to-plant variation [4], [13] and [28]. However, decrease of L-GalLDH action by RNA interference in plant, which contributed to decreased L-ascorbate rates, did not change the L-ascorbate production capability in the transgenic green plants. It appears that L-GalLDH plays a key role in the control of L-ascorbatelevel by light; the mechanism is vague but may possiblyparticipate light-dependent regulation of L-GalLDHexpression and also light-dependent variations in respiration that can directly act onL-GalLDHfunction [3] and [29].There is no AA concentration without L-GalLDH because the final reaction in the L-ascorbatepathway is oxidation of L- L-galactono-1, 4-lactone (GalL) to AA (*Figure: 4*). This reaction is catalysed by L-galactono-1, 4-lactone dehydrogenase (L-GalLDH). This uses C-type Cytochrome *c* (*Cyt c*) as its electron acceptor [15], [28] and [29]. the steps of L-ascorbate pathway occur in different compartments of the plant cell. Earlier studies indicated that L-galactose is a comparatively rare sugar; whereas, this is famous to occur in small quantities in the cell wall of vascular plants. Moreover, this is a module of polysaccharides in some animals and eukaryotic algae. This step is occurred in the compartment of Cytosol, but the terminal step which is occurred in Mitochondria is the oxidation of 2 an oxidized c-type cytochrome + L-GalL to AA + 2 areduced c-type Cytochrome + $3H^+$ catalyzed by L-GalLDH at C2 or C3. C-type cytochrome which is located on the inner mitochondrial membrane is used as electron acceptor [30] and [32]. They showed L-GalLDH to be famous to occur in Mitochondria in association with respiratory complex I [33].However, there is a difference between the final steps of L-ascorbate biosynthesis in the kingdoms plants, fungi and animals (Table-1).

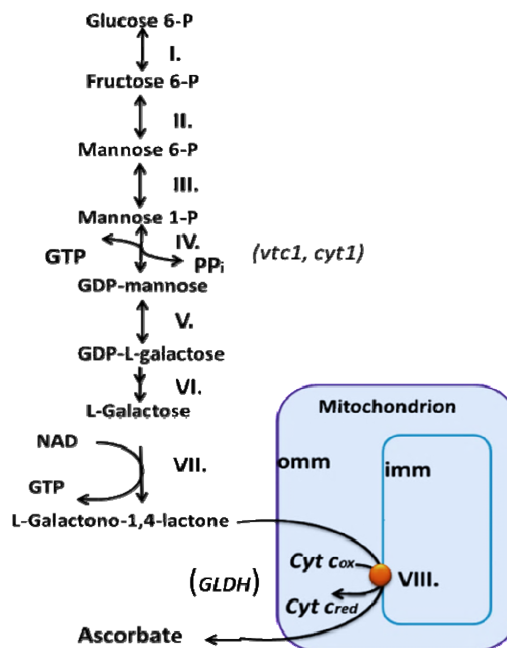

Figure-4: The L- ascorbate biosynthesis in green plants such as *Arabidopsis thatliana*. Enzymes:(I) glucose-6-phosphate isomerase [EC:5.3.1.9]; (II) mannose-6-phospho isomerase [(Gene: AT3G02570.1 and AT1G67070.1) EC:5.3.1.8]; (III) phosphomannomutase (Gene: AT2G45790.1 and AT1G70820.1) [EC:5.4.2.8]; (IV) mannose-1-phosphate guanylyltransferase (Gene: VTC1 or AT2G39770.1) [EC:2.7.7.13]; (V) GDP-D-mannose 3', 5'-epimerase (Gene: AT5G28840.1) [EC:5.1.3.18]; (VI) GDP-L-galactosephosphorylase (Gene:AT5G55120.1 and AT4G26850.1) [EC:2.7.7.69]; (VII) L-galactose dehydrogenase (Gene: AT4G3670.1, L-galDH) [EC:1.1.1.316]; and (VIII) L-galactono-1,4-lactone dehydrogenase (Gene: GLDH or AT3G47930.1, L-galLDH) [EC:1.3.2.3]. The final oxidation of L-galactono-1,4-lactone to AA occur on the inner mitochondrial membrane uses c-type cytochrome as an electron acceptor and is therefore coupled to mitochondrial electron transport [28].

Tabel-1: Comparison of enzyme involved in the final step in the biosynthetic pathway of either ascorbate or erythroascorbic acid in the three kingdoms.

Features	*Plant*	*Fungi*	*Animal*
Example	*Arabidopsis thaliana, Solanumtuberosum, Spinach, and Cauliflower enzymes.*	*Saccharomyces cerevisiae and Candida albicans enzymes.*	*chicken and rat enzymes.*
Enzyme	*L-Galactono-1, 4-Lactone Dehydrogenase (L-GalLDH)*	*AraL Oxidase (AraLO)*	*L-gulonolactone oxidase (L-gulLO)*
E.C. Number	*1.3.2.3*	*1.1.3.24*	*1.1.3.8*
Subcellular-compartment	*Mitochondria*	*Mitochondria*	*Microsomes*
Product	*Ascorbate*	*D-Erythroascorbic acid*	*Ascorbate*
Prosthetic group	*Non-covalent flavin*	*Covalent flavin*	*Covalent flavin*
Electron acceptor	*Cytochrome c*	*O2*	*O2*
L-Galactone-1,4-lacton	*100*	*87*	*87*
D-AraL	*0*	*100*	*0*
L-GuIl	*0-20*	*24*	*100*
D-Xylonolactone	*0*	*85*	*-**
Native molecular mass (KD)	*56*	*51*	*56-60*

-*= undetermined

Material and Method

1- Plant materials

In a laboratory experiment, the species of five different plant classes were investigated in order to determine the presence or absence of an activity L-GalLDH. Vascular plants are green plant, Bryophytes (mosses) and Pteridophytas (ferns) but non vascular plants areChlorophytes (green algae),Phaeophyceaes (brown algae)andRhodophyta (red algae). From the vascular plant, *Pisumsativum*was taken as a control because there is evidence that it contains the L-GalLDH enzyme. However, three species of the Chlorophytes were collectedin the sea; *Blindingia minima,Caulerpa*and*Ulvalactuca;* while,the class Phaeophyceae included *Hesperophycuscalifornicus, Fucusgardneri, Ascophyllumand Chlorodesmis*. The class Rhodophyta included *Palmariapalmata, Porphyrapurpurea*and *Gracilaria*. On the other hand, the Bryophyte species included: *Orthotrichumcrassifolium, Bryumcapillare*and *Tortulamuralis* and also the Pteridophytaspecies:*Aspleniumscolopendrium, Aspleniumtrichomanes*and *Athrium optophorum.* The members of mosses and ferns were collected in facilities belonging to the University of Exeter. There was no evidence about whether these species of Bryophyte, Pteridophyta, Phaeophyceae, *Rhodophyta* and Chlorophyte contain the L-GalLDH enzymes or not, so that we will investigate some species of these lineages to determine the presence or absences of an activity of the L-GalLDH.

2- Subcellular Fractionation

The following procedures were carried out at 4°C. In this experiment, The plant leaves of each sample were homogenized in extraction medium (30 mM 3-[*N*morpholino]-propanesulfonic acid [MOPS], pH 7.5, 0.2% [w/v] bovine serum albumin [BSA], 4 mMCys, 0.35 Mmannitol, 1% [w/v] PVP 40, 25 mM $Na_4P_2O_7$, and 2 mM EDTA) at an amount of 1 gm of leave tissue per 5 mL of extraction medium, in a homogenizer. The homogenate could be cleaned via 4 layers of cotton cloth and centrifuged at 2,200gfor 5 min to attain the chloroplast fraction. This was re-suspended in 10 mL of washing medium (20 mM MOPS, pH 7.2, 0.2% BSA [w/v], 0.3 Mmannitol, and 1 mM EDTA), and centrifuged at the same speed once more. The first 2,200gsupernatant was centrifuged at 14,000gfor 15 min to produce a fraction enriched in Mitochondria. this

pellet in washing medium was re-suspended, and centrifuged again at 14,000gbefore being loaded onto a gradient including a lower layer of 15 mL of 28% (w/v) Percoll in 10 mM MOPS, pH 7.2, 0.3 M Sucrose, and 0.1% (w/v) BSA, and an upper layer of 21 mL of the same solution with mannitol instead of Sucrose***[33]***. After centrifugation at 41,400g for 35 min, intact Mitochondria were washed twice by a 10-times dilution in washing medium and centrifugation at 17,400gfor 15 min.

3- Separation of Outer and Inner Membranes of Mitochondria

As explained by Mannella (1987), we isolated Outer- and inner-membrane-enriched fractions from Mitochondria of plant leaves***[34].*** In this approach, unbroken Mitochondria are exposed to hypotonic shocks, which boosts GalLDH activities because both outer- and inner- membranes of Mitochondria are being disturbed. These unbroken Mitochondria isolated from leaves. These were then swollen in 12 mMmannitol for 30 min, a content is appropriate to disperse the outer membrane without disturbing the inner membrane. After that, the unbroken Mitochondria are put in a 0.6 to 0.9 M Sucrose-step gradient, which was centrifuged at 40,000gfor 60 min. Outer-membranesof Mitochondria were remained at the supernatant fraction between the lower layer and the Sucrose interphase. From the bottom of the tube, remaining intact Mitochondria and Inner membranes were assembled. At least three times in a 20 mM MOPS, pH 7.2, and 0.3 Mmannitol, and centrifuged at 60,000gfor 1.5 h, both fractions were diluted. Pellets making of outer- and inner-membrane of Mitochondria enriched fractions were re-suspended in small volumes of the same medium.

4- Enzyme assay methods

The L-GalLDH enzymes were extracted using the procedure as explained by Bartoli et al (2000). The biochemical assays are conducted according to the strategy of the following examiners [31] and [32]**.** This enzyme was read by Tecan spectrophotometer.

5- Preparation of microplate reader

Microplate reader (known as plate reader) is a necessary instrument for observing biological event of samples in absorbance reader. For each enzyme, a certain volume of different buffer solution was added together with 5 to 10 µl of extracts that was added to a plate well to read the activity of the L-GalLDH.

6- Absorbance reading for preparing samples

TECAN spectrophotometer is a sensitive, accurate, reproducible, fast and flexible system. This system is capable of reading multiple sample materials during a limited time. This system was used to measure the absorbance of the L-GalLDH activity. The settings for measuring the absorbance of were at 25°C and 340 nm for 60 min. Furthermore, this type of a spectrophotometer is capable of calculating the absorbance sample that passed through the sample solution.

Results

L-GalLDH has played a key role in catalyzing of the terminal step of L-galactose pathway. This is proved to have the similar functionin flowering and non-flowering plants [22] and [33]. An investigation of the activity of L-GalLDH (EC:1.3.2.3) in some species of flowering and non-flowering plants was a key focus of this experimental study. this enzyme was determined in five classes; higher plant,Chlorophyte, Phaeophyceae, Bryophyte and Pteridophyte (Table-2). In each class, more than one species was tested, only in flowering plants, *Pisumsativus* was considered for the experiment. It reported that the function of this enzyme from *Pisumsativus* is the same other green plants because It contains a high content of this enzyme [20]. It was unpredictable to confirm that the activity of L-GalLDH is present in the species of Chlorophyta, Rhodophyta,Bryophyta, Pteridophyta and Phaeophyceae. In this experiment, it was proved that *Blindingiaminima* which belongs to chlorophyte contained a low concentration of L-GalLDH. This result is consistentwith a previous study becausethis species can produce a low content of AA [33].

Furthermore,*Porphyra purpurea* which belongs to Rhodophyta has a very low content of this.However, other species of both Rhodophyta and Chlorophyta did not contain L-GalLDH's activity. This result is consistent with current investigations. Some species of eukaryotic algae cannot produce vitamin c. The pathway of ascorbate biosynthesis of red seaweed has been recentlyfound. it can possibly be suggested that the pathway of red seaweeds could be similar to those in flowering plants. However, it can be a completely new route that has not been found. Thus, it is hypothesised that the pathway is similar to flowering plants.However, the members of Bryophyte, Phaeophyceae and Pteridophyta did not possess the activity of L-GalLDH. However, the ascorbate pathway of the species of Bryophyte and Phaeophyceae have not yet been discovered, it can be an alternative route that has not been discovered.However, it can be suggested that the ascorbate pathway of these is similar to flowering plants.

Table-2: Showing presence and absence of L-GalLDH activity in the species of five classes of photosynthetic eukaryotes.

Photosynthetic plants classes		
Plant name	**Species name**	**L-GalLDH**
Higher plant	*Pisumsativum*	*Yes*
Chlorophyte (green algae)	*Blindingia minima*	*Yes*
	Ulvalactuca	*No*
	Caulerpa	*No*
Phaeophyceae (brown algae)	*Fucusgardneri*	*No*
	Hesperophycuscalifornicus	*No*
	Chlorodesmis	*No*
	Ascophyllumnodosum	*No*
Bryophyte (moss)	*Orthotrichumcrassifolium*	*No*
	Bryumcapillare	*No*
	Tortulamuralis	*No*
Pteridophyte (ferns)	*Aspleniumscolopendrium*	*No*
	Aspleniumtrichomanes	*No*
	Athrium optophorum	*No*
Rodophyte (red algae)	*Palmaria palmata*	*No*
	Porphyra purpurea	*Yes*
	Gracilaria	*No*

Conclusion

L-GalLDH enzyme is involved in the last step ofascorbate biosynthesis pathway from photosynthetic eukaryotes. In the present study, molecular phylogenetic analysis is used to infer the historical and evolutionary relationships between homologous sequences of it. First of all, the phylogenetic tree result reveals that there is an historical relationship among the pathway of ascorbate biosynthesis of flowering and non-flowering plants. Secondly, ML tree shows that the events of gene evolution can result in the evolution of the L-GalLDH enzyme among the lineages of photosynthetic eukaryotic organisms. One of the evolutionary events is gene duplication that has played a pivotal role in the evolution of multiple paralogous copies of these enzymes in a species called within species in-paralogs and in different species called between-species out-paralogs. Another events is speciation that can lead to the evolution of orthologs in different species. Thirdly, the phylogenetic tree showed that it mostly occurs in vascular plants, in comparison with non-vascular plants. These enzymesare more broadly evolved and diverged from their common ancestors. Fourthly, biochmical experiments were conducted to observe the activity of L-GalLDH enzyme from five classes of photosynthetic eukaryotes. Within these classes there is only two species of Chlorophyta (*Blindingia minima*) and Rhodophyta (*Porphyra purpurea*) had a very low concentration of this enzyme, compared to higher plants (*Pisumsativum*). Other species of the classes Rhodophyta, Chlorophyta, Bryophyte, Phaeophyceae and Pteridophyta did not contain the L-GalLDH activity.it is hypothesized thatthe L-ascorbate pathwayin these speciescan be a completely new routethat has not been discovered. However, it

can possibly be suggested that the pathway is similar to flowering plants. Finally, after a closer examination of the comparative protein trees with species trees, it can be suggested with a significant degree of certainty that paralogs and orthologs of each enzyme can provide useful evidence and information on evolutionary relationship and taxonomic classification of molecules and photosynthetic eukaryotes. Furthermore,this now opens the gate for understanding how these enzymes are distributed and sequentially similar.

References

[1] Mapson, L. W. and Breslow, E. "*Biological synthesis of ascorbic acid: L-galactono-1,4-lactone dehydrogenase*", Biochemical Journal, Vol.68, pp. 395–406. (1958).

[2] Smirnoff, N., Conklin, P. L. and Loewus, F. A. "*Biosynthesis of ascorbic acid in plants: A renaissance. Annual Review of Plant Physiology and Plant Molecular Biology*", Biochemical Journal, Vol.52, pp. 437–467. (2001).

[3] Millar, A. H. Mittova, V. Kiddle, G. Heazlewood, J. L., Bartoli, C. G. Theodoulou, F. L. and Foyer, C. H. "*Control of ascorbate synthesis by respiration and its implications for stress responses*", Plant Physiology, Vol.133, pp. 443–447,(2003).

[4] Ishikawa, T. and Shigeoka, S. "*Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms*", Bioscience, Biotechnology, and Biochemistry,Vol.72, pp. 1143–1154, (2008).

[5] Hancock, R. D. and Viola, R. "*The use of micro-organisms for L-ascorbic acid production: Current status and future perspectives*", Applied Microbiology and Biotechnology,Vol.56, pp. 567–576.(2001).

[6] Hancock, R. D. and Viola, R. "*Biotechnological approaches for L-ascorbic acid production*" Trends in Biotechnology,Vol.20, pp. 299–305.(2002).

[7] Hancock, R. D., Walker, P. G., Pont, S. D. A., Marquis, N., Vivera, S., Gordon, S. L., Brennan, R. M. and Viola, R. "*L-Ascorbic acid accumulation in fruit of Ribesnigrum occurs by in situ biosynthesis via the L-galactose pathway*" Functional Plant Biology,Vol.34, pp. 1080–1091.(2007).

[8] Alhagdow, M., Mounet, F., Gilbert, L., Nunes-Nesi, A., Garcia, V., Just, D., Petit, J., Beauvoit, B., Fernie, A. R., Rothan, C. and Baldet, P. "*Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase affects plant and fruit development in tomato*", Plant Physiology,Vol.145, pp. 1408–1422.(2007).

[9] Pineau, B., Layoune, O., Danon, A. and de Paepe, R. "*L-Galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I*". Journal of Biological Chemistry,Vol.283, pp. 32500–32505.(2008).

[10] Shigeoka, S., Nakano, Y. and Kitaoka, S. "*Biosynthetic pathway of L-ascorbic acid in Euglena gracilis Z*", Journal of Nutritional Science and Vitaminology,Vol.25, pp. 299–307.(1979).

[11] Helsper, J. P., Kagan, L., Hilby, C. L., Maynard, T. M. and Loewus, F. A. "*L-Ascorbic acid biosynthesis in Ochromonasdanica*", Plant Physiology,Vol.69, pp. 465–468.(1982).

[12] Grun, M. and Loewus, F. A. "*L-Ascorbic acid biosynthesis in the euryhaline diatom Cyclotellacryptica. Planta*", Vol.160, pp. 6–11.(1984).

[13] Leferink, N. G. H., van den Berg, W. A. M. and van Berkel, W. J. H. "*L-Galactono-gamma-lactone dehydrogenase from Arabidopsis thaliana, a flavoprotein involved in vitamin C biosynthesis*", The FEBS Journal, Vol.275, pp. 713–726.(2008).

[14] Ostergaard, J., Persiau, G., Davey, M. W., Bauw, G. and Van Montagu, M. "*Isolation of a cDNA coding for L-_-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants—Purification, characterization, cDNA cloning, and expression in yeast*", Journal of Biological Chemistry, Vol.272, pp. 30009–30016.(1997).

[15] Huh, W. K., Kim, S. T., Yang, K. S., Seok, Y. J., Hah, Y. C. and Kang, S. O. "*Characterization of D-arabinono-1,4-lactone oxidase from Candida-albicans Atcc-10231*" European Journal of Biochemistry Vol.225, pp. 1073–1079.(1994).

[16] Baroja-Mazo, A., del Valle, P., Rua, J., de Cima, S., Busto, F., de Arriaga, D. and Smirnoff, N.

"*Characterisation and biosynthesis of D-erythroascorbic acid in Phycomycesblakesleeanus*", Fungal Genetics and Biology,Vol.42, pp. 390–402.(2005).

[17] Amako, K., Fujita, K., Shimohata, T. A., Hasegawa, E., Kishimoto, R. and Goda, K. "*NAD-specific D-arabinose dehydrogenase and its contribution to erythroascorbic acid production in Saccharomyces cerevisiae*" FEBS Letters,Vol.580, pp. 6428–6434.(2006).

[18] Smirnoff N. "*Ascorbic acid: metabolism and functions of a multi-facetted molecule",*European Journal of Plant Biology,Vol.3, pp. 229-235.(2000).

[19] Jones, T. Taylor, R., and Thornton, M. "*The rapid generation of mutation data matrices from protein sequences*", Computer Applications in the Biosciences,Vol.8, pp. 275-282.(1992).

[20] Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. "*MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.",*Molecular Biology and Evolution, Vol.30, pp. 2725-2729.(2013).

[21] Keeling, J. Burger, G.Durnford, G. Lang, F. Lee, W. Pearlman, E. Roger, J. and Gray, W."The tree of eukaryotes", TRENDS Ecological EvolutionVol. 20, pp. 670-676.(2005).

[22] Bhattacharya, D. and Medlin, L."*The phylogeny of plastids: a review based on comparisons of small-subunit ribosomal RNA coding regions*", Journal of Phycololgy, Vol.31, pp. 489-498.(1995).

[23] Rodriguez-Ezpeleta, N.Brinkmann, H. Burey, C.Roure, B. Burger, G. Loffelhardt, W. Bohnert, J. Philippe, H. and Franz Lang, B."*Monophyly of primary photosynthetic eukaryotes: Green plants, Red algae, and Glaucophytes*", Journal of Current Biology, Vol.15, pp. 1325-1330.(2005).

[24] Miller, G. Suzuki, N. Rizhsky, L. Hegie, A. Koussevitzky, S. and Mittler, R. "*Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses*". Plant Physiology,Vol.144, pp. 1777–1785. (2007).

[25] Stiller, W. and Hall, D."*The origin of red algae: implication for plastid evolution*", Proceeding of the National Academy Science,Vol.94, pp.4520-4525.(1997).

[26] Burger, G. Saint-Louis, D. Gray, W. and Franz Land, B."*Complete sequence of the mitochondrial DNA of the red alga Porphyrapurpurea: cyanobacterial introns and shared ancestry of red and green algae*", Journal of purnal of Plant Cell, Vol.11, pp. 1675-1694.(1999).

[27] Moreira, D. Le Guyader, H. and Philippe, H."*The origin or red algae and the evolution of chloroplasts*", Journal of Nature Vol.405, pp. 69-72.(2000).

[28] Smirnoff, N. "*L-Ascorbic acid biosynthesis. Vitamins and Hormones",*Vol.61, pp. 241–266.(2001).

[29] Smirnoff, N. and Gatzek, S. "*Ascorbate biosynthesis: A diversity of pathways. In Vitamin C. Function and Biochemistry in Animals and Plants*", Bioscience Scientific Publishers, Oxford, Vol.34, pp. 7–29. (2004).

[30] Loewus F., A., Jang R and Seegmiller C., G."*Conversion of C-14 labeled sugars to L-ascorbic acid in ripening strawberries*"Journal of Biological Chemistry, Vol.222, 649–664.(1956).

[31] Wheeler GL, Jones MA and Smirnoff N."*The biosynthetic pathway of vitamin C in higher plants*",Journal of Nature,Vol.393, pp. 365-369.(1998).

[32] Bartoli, G., Pastori, M. and Foyer H."*Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV*", Journal of Plant Physiology,Vol.123, pp. 335-343, (2000).

[33] Jime´nez, A.Herna´ndez, A. del Rı´o A.and Sevilla, F."*Role of ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves*",Journal of Plant PhysiologyVol.118, pp. 1327–1335.(1997).

[34] Mannella, A."*Isolation of the outer membrane of plant mitochondria*", Methods Enzymology Journal,Vol.148, pp. 453–464.(1987).