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Biochemical and Biotechnological Perspectives of GDP-L-Galactose Phosphorylase, L-galactose Dehydrogenase, L-Galactono-1,4-Lactone Dehydrogenase: A Review

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ABSTRACT: L-ascorbic acid (vitamin C) is an essential water-soluble antioxidant and acts as an enzymes cofactor involved in the biosynthesis of necessary molecules. It is requisite for both plants and animals. In the past two decades, metabolic pathways and genetic regulation of ascorbate biosynthesis have been explored. A plethora of studies proposed that the Smirnoff-Wheeler pathway contributes to the major content of ascorbic acid. So far, attempts have been made to regulate metabolic pathways by cloning and overexpressing genes of biosynthetic pathways to enhance ascorbic acid content. The diversity and variability of L-ascorbic acid among different plant tissues remain the challenge. Thus, in this review, gene cloning strategies of GGP (GDP-L-Galactose Phosphorylase), GalDH (L-galactose Dehydrogenase), GLDH (L-Galactono-1,4-Lactone Dehydrogenase), and consequences of their overexpression or suppression on ascorbate content and overall plant growth including tolerance has been discussed. Overexpression of GGP and GLDH in model plants showed reasonable results. However, stable translation of these results in consumable crops to improve nutritional superiority will necessitate a novel targeted approach. Moreover, additional interventions are being explored so that can be employted to enhance the L-ascorbic acid content in different species of crop by using different molecular approaches.

Keywords: Ascorbic acid, Vitamin C, L-galactose dehydrogenase, GDP-L-Galactose Phosphorylase, L-Galactono-1,4-Lactone Dehydrogenase, Gene cloning, ascorbate biosynthesis.

INTRODUCTION

L-Ascorbic acid (L-AA) ($C_6H_8O_6$), otherwise designated as vitamin C is a six-carbon sugar molecule derived from l-threo-hex-2-enono-1,4-lactone. L-AA is a well-known water-soluble antioxidant synthesized by both plants and animals; however, humans and some mammals cannot synthesize ascorbate because of mutation in the gene coding for terminal enzymeL-galactono-1,4-lactone dehydrogenase in the biosynthetic pathway;thus it becomes an essential dietary supplement (Nishikimi *et al.,* 1994; Tareen *et al.,* 2015; Kaur *et al.,* 2021). The ascorbic acid sourcesfor humans include fresh fruits (orange, grapes, strawberries, papaya, lemon, cherries, watermelon, gooseberry, etc.) and vegetables (broccoli, peppers, cauliflowers, etc.). Ascorbate is usually present at different concentrations in different fruits and vegetables, andthe utmost concentration is accorded in Indian gooseberry (*Phyllanthus emblica*).The following table encloses the concentration of vitamin C in some frequent edible fruits and vegetables.

Ascorbate is involved in numerous cell signaling and physiological processes; it acts as a cofactor for metalloenzymes, anti-oxidative metabolites, electron acceptors, and donors (Nehzo *et al.,* 2015; Panahi and Dehdivan, 2017). In animals, ascorbate regulates collagen, carnitine, and neurotransmitters biosynthesis by regulating enzymes hydroxylase and monooxygenase (Hulse *et al.,* 1978), necessary for immune cell functioning, iron consumption, and cardiovascular function. Recent evidences have shown the protective effect of ascorbate in coronary heart disease, anti-cancer properties, and direct rapport with bone mineral density (BMD) (Hancock and Viola., 2002; Wolf *et al.,* 2005). In plants, ascorbate mainly protects reactive oxygen species (ROS) and controls cell growth and expansion by regulating enzyme prolyl hydroxylase (Smirnoff and Wheeler, 2000).

Table 1: Common edible fruit and vegetable with vitamin C content.

Plant	Concentration of Vitamin C (in mg/100g)	Reference
Citrus X sinensis (Orange)	43-58mg/100gm	Najwa and Azrina (2017)
Actinidia deliciosa (Kiwi)	91mg/100gm	Dumbrava *et al*. (2016)
Citrus limon (Lemon)	31-44mg/100gm	Najwa and Azrina (2017)
Ananas comosus (Pineapple)	45mg/100gm	Dumbrava *et al*. (2016)
Fragaria × *ananassa* (Strawberry)	31-54mg/100gm	Zhong *et al.* (2016)
Citrus × *paradise* (Grapefruit)	26-49mg/100gm	Najwa and Azrina (2017)
Brassica oleracea (Brocolli)	106-117mg/100gm	Vallejo *et al.* (2002)
Phyllanthus emblica (Amla)	400-600mg/100gm	Jain and Khurdiya (2004)
Punica granatum (Pomegranate)	23-30mg/100gm	Dumbrava *et al*. (2016)

Four pathways are explored for ascorbate biosynthesis in plant cells, namely, Smirnoff-Wheeler, Myo-inositol, L-glucose, and galacturonic. The most extensively studied pathway is the Smirnoff-Wheeler/L-galactose pathway; all the enzymes and substrates of this pathway have been well characterized. A comprehensive study of the ascorbate biosynthetic pathway provides a gateway for controlling the ascorbate concentration in plant cells. However, only knowing the enzyme and pathway would not be sufficient to manipulate ascorbate biosynthesis. The factors and cofactors involved with enzymes also play a significant role.

This study mainly focuses on enzymes of the L-galactose pathway and their biotechnological applications for enhancing ascorbate synthesis. The L-galactose pathway is a 10-step metabolic process in plant cells. D-glucose is the initial substrate that gets converted into L-galactose in the first eight steps. L-galactose is ultimately converted into L-ascorbic acid in a two-step process (Wheeler *et al.,* 1998). Initially, D-glucose-6-P is converted into GDP-D-mannose via intermediate D-fructose-6-P, D-mannose-6-P, and D-mannose-1-P; further, GDP-D-mannose is converted into L-galactose by conversion of GDP-D-mannose to GDP-L-galactose which is catalyzed by enzyme GDP-D-Man-3'-5'-epimerase, then GDP-L-galactose is converted into L-galactose by the action of GDP-L-gal phosphorylase and L-gal 1-P phosphatase. L-galactose is oxidized to L-galactono-1,4-lactone by NAD-dependant L-galactose dehydrogenase. L-galactono-1,4-lactone is a valuable precursor for the synthesis of L-ascorbic acid. A mitochondrial enzyme, L-galactono-1,4-lactone dehydrogenase acts upon L-galactono-1,4-lactone and directly converts it into L-ascorbic acid (Wheeler *et al.,* 1998; Gallie., 2013; Ishikawa *et al.,* 2018).

BIOSYNTHESIS OF ASCORBIC ACID

Ascorbic acid biosynthetic pathway has been a major research gap for years; in the 1950s, ascorbate biosynthesis was first examined. After 40 years, Wheeler et al. (1998) demonstrated the first biosynthetic pathway with all known intermediate metabolic products and enzymes.

At least four pathways have been described for ascorbate biosynthesis in plants, including Smirnoff-Wheeler, Myo-inositol, L-gulose,and galacturonic. Mammalian ascorbate biosynthetic pathway differs from plants; D-glucose is the initial substrate in mammals which gets converted into D-glucuronic acid. Conversion of D-glucose into D-glucuronic acid is a five-step process involving intermediate D -glucose-6-P, D-glucose-1-P, UDP-D-glucose, UDP-D-glucuronic acid D-glucuronic acid-1-P respectively; these steps are catalyzed by the enzyme phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, glucuronate-1-phosphate uridylyltransferase, glucuronate 1-kinase respectively. Further L-ascorbic acid is produced from D-glucuronic acid via two intermediates, L-gulonic acid, and L-gulono-1,4-lactone; these two steps are catalyzed by glucuronate reductase, aldonolactonase, and gulono-1,4-lactone oxidase, respectively (Burns, 1967; Nishikimi and Yagi, 1996).

Earlier studied pathways in plants suggested the inversion of the carbon chain for biosynthesis from D-galacturonic acid derivatives. Later, it was proved incorrect by nourishing plant cells with labeled C6 sugar, which suggested no inversion of carbon backbone (Loewus, 1963; Smirnoff and Wheeler, 2000). In plants, the major ascorbate biosynthetic pathway is the GDP-mannose/L-galactose pathway which suggests no inversion of the carbon skeleton and involves two key metabolites, i.e., D-mannose-6-P and L-galactose. To support the hypothesis that mannose and L-galactose are key metabolites of the pathway, they fed labeled (^{14}C) mannose to *Arabidopsis* leaf extract, and 4h incubation resulted in accumulation of about 10% ascorbate with labeled carbon, and feeding L-galactose to *Arabidopsis* leaves results in enhanced production of ascorbic acid (Wheeler *et al.,* 1998). However, Davey and the group confirmed the role of L-galactose in the pathway, and they reported a rapid increase in ascorbic acid content when L-galactose was supplied exogenously (Davey *et al.,* 1999).

Synthesis of ascorbate without inversion requires three main steps, first oxidation of carbon 1, which ultimately results in the formation of L-galactono-1,4-lactone, second oxidation of carbon 2 and 3 to form unsaturated

diol, and third epimerization step to change D configuration to L configuration at carbon 5 (Loewus *et al.,* 1987; Loewus, 1999). Smirnoff-Wheeler pathway starts with PGI (phosphoglucose isomerase) enzyme activity that converts D-glucose-6-phosphate to D-fructose-6-phosphate; further D-fructose-6-phosphate isomerizes into D-mannose-6-phosphate by PMI (phosphomannose isomerase). In *Arabidopsis*, PMI1 expression and increase in ascorbic acid content are directly comparative under light conditions, whereas knock-down PMI1 and dark conditions are associated with a decrease in ascorbic acid concentration (Maruta *et al.,* 2008). PMM (phosphomannose mutase) then converts D-mannose-6-phosphate into D-mannose-1-phosphate. The activity of PMM has been shown in *Arabidopsis* and *Nicotiana benthamiana* (Hoeberichts *et al.,* 2008; Qian *et al.,* 2007).

Guanosine monophosphate of GTP is added to D-mannose-1-phosphate by GMP (GDP-D-mannose-pyrophosphorylase) to form GDP-D-mannose. Gene VTC1 codes for GMP in *Arabidopsis*, VTC1 mutants produce only 25-30% of ascorbic acid compared to wild type and established sensitivity to ROS such as H_2O_2, SO_2, etc. (Conklin *et al.,* 1999; Keller *et al.,* 1999). GME (GDP-D-mannose epimerase) epimerizes GDP-D-mannose to form GDP-L-galactose,the most conserved enzyme in ascorbate biosynthesis; silencing of GME gene demonstrated the reduced ascorbate content in tomato, which had a direct impact on plant growth and development (Voxeur *et al.,* 2011). In contrast, overexpression of GME resulted in increased accumulation, which is also positively associated with abiotic stress tolerance (Zhang *et al.,* 2013). GME also epimerizes GDP-D-mannose to GDP-L-gulose, a precursor of an alternative pathway (Wolucka and Montagu, 2007). GGP (GDP-L-galactose-phosphorylase) catalyzes the conversion of GDP-L-galactose to L-galactose-1-phosphate, which is the first dedicated step of the Smirnoff-Wheeler pathway (Bulley and Laing, 2016). GGP1 mutant containing tomato plants exhibit significantly less ascorbate accumulation in leaves, roots, flowers, and fruit; ultimately, GGP1 mutant affects the yield of tomatoes, suggesting their role in ascorbate synthesis and direct sunlight impact on yield (Alegre *et al.,* 2020). VTC2 and VTC 5 genes code for GGP in *Arabidopsis*; mutant VTC2 and VTC5 *Arabidopsis* were unable to survive unless nourished with galactose or ascorbate, suggesting the necessity of active GGP for ascorbate production (Dowdle *et al.,* 2007). VTC4 in *Arabidopsis* codes for GPP (L-galactose-1-phosphate phosphatase), which converts L-galactose-1-phosphate into L-galactose. GPP is a metalloenzyme, and its activity is driven by Mg2+ (Cocklin *et al.,* 2006). L-galactose oxidizes into L-galactono-1,4-lactone in the presence of NAD-dependent GalDH (L-galactose dehydrogenase) (Wheeler *et al.,* 1998). The last step of this pathway is catalyzed by terminal enzyme GLDH (L-galactono-1,4-lactone dehydrogenase), which is present in the inner membrane of mitochondria. GLDH catalyzes the conversion of L-galactono-1,4-lactone into L-ascorbic acid (Smirnoff *et al.,* 2001). RNAi silenced GLDH results in decreased ascorbate pool and overall affects the growth rate of the tomato plant (Alhagdow *et al.,* 2007).

Loewus and Kelly in strawberry fruit describe another biosynthetic pathway of L-ascorbic acid; to check the biosynthesis of ascorbic acid, they have utilized D-galacturonic acid with radiolabelled C (^{14}C), D-galacturonic acid is further converted into L-ascorbic acid-6-^{14}C. In this pathway, pectin (a component of the plant cell wall) is breakdown into D-galacturonic acid, which is further reduced by an NADPH-dependent enzyme GalUR (Galacturonic acid reductase) to form L-galactonic acid. Aldonolactonase acts upon L-galactonic acid and converts into L-galactono-1,4-lactone, a terminal intermediate molecule formed in the Smirnoff-Wheeler pathway that ultimately metabolizes into L-ascorbic acid by the action of an enzyme GLDH (Loewus and Kelly, 1961; Gallie, 2013). Researchers have also shown increased level of expression of GalUR is responsible for the augmented production of L-ascorbic acid (Agius *et al.,* 2003).

An extensive case study of ascorbate biosynthetic pathways in *Arabidopsis* has shown that GME can potentially transform GDP-D-mannose to GDP-L-galactose by 3'-5' isomerase activity along with GDP-L-gulose by 5' isomerase activity. GDP-L-gulose is an initial substrate for the L-gulose pathway, which is further sequentially converted into L-gulose-1P, L-gulose, and L-gulono-1,4-lactone. L-gulono-1,4-lactone can then be converted into L-ascorbate. However, the enzyme responsible for catalyzing the conversion of L-gulono-1,4-lactone to L-ascorbate was unknown till 2017 (Wolucka, Vam Montagu, 2003; Maruta *et al.,* 2010). Feeding L- gulono-1,4-lactone has increased the ascorbate content in multiple plants, Aboobucker *et al.* (2017) have successfully purified the enzyme catalyzing the last step of the L-gulose pathway. The enzyme GulLO (L-gulono-1,4-lactone oxidase) (FAD dependant) oxidized L-gulono-1,4-lactone to L-ascorbate and was purified from *Arabidopsis* transient expression system when they feeded*N. benthamiana* expressing recombinant AtGulLO5 gene with L-gulono-1,4-lactone ascorbate; concentration increased as compared to control (Aboobucker *et al.,* 2017).

Incubation of D-glucuronic acid and L-gulono-1,4-lactone in *Arabidopsis* suspension culture showed increased accumulation of ascorbic acid. The potential precursor of the D-glucuronic pathway is Myo-inositol; in this pathway,Myo-inositol is oxidized into D-glucuronic acid by MIOX (Myo-inositol oxygenase), GlcUR (glucuronate reductase) further convert D-

glucuronic acid into L-gulonic acid which sequentially transformed into L-gulono-1,4-lactone and L-ascorbic acid by AL (aldonolactonase) and LGulDH (L-gulonolactone dehydrogenase), respectively (Lorence *et al.,* 2004). They have cloned the gene coding for MIOX and expressed it in E.coli to check the role of Myo-inositol in ascorbate biosynthesis. RNA-gel blot analysis showed a 2-3 fold increased expression of MIOX in flowers and leaves of Arabidopsis.

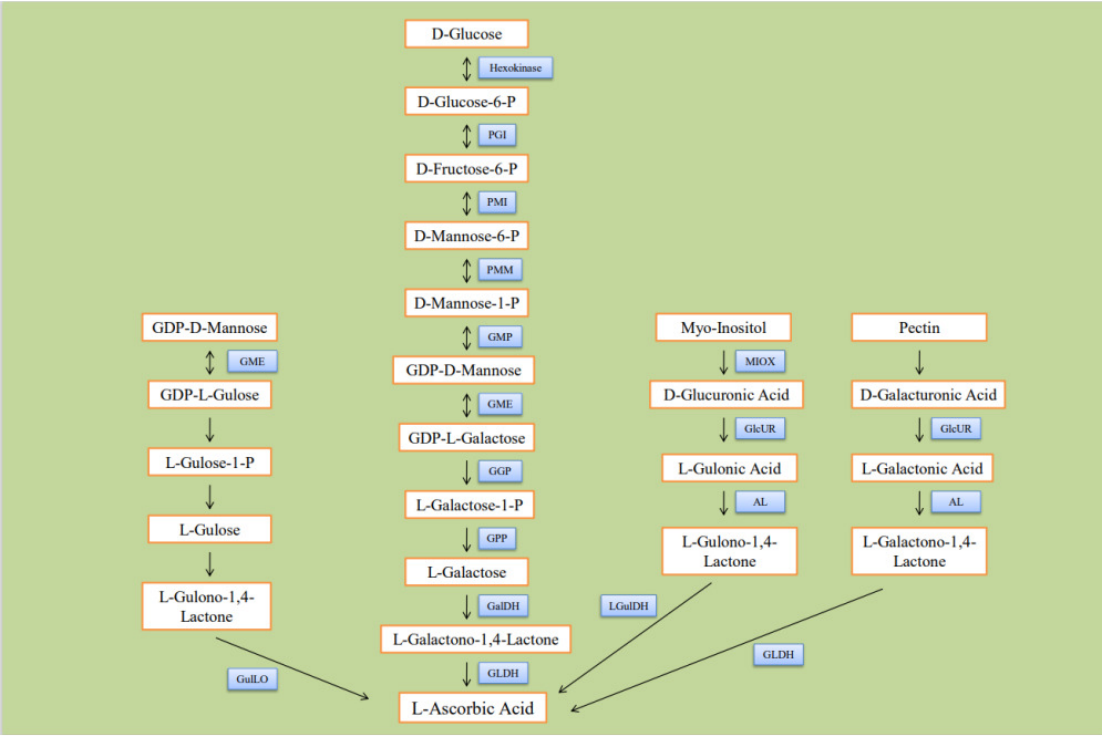

Fig. 1. L-ascorbate biosynthetic pathway, namely L-gulose pathway, Smirnoff-Wheeler pathway, Myo-inositol pathway, and Galacturonic pathway (left to right). Abbreviated name of enzymes catalyzes the metabolic reaction, which is shown in blue colored rectangular boxes, GME (GDP-D-Mannose-3′,5′-Epimerase), GulLO (L-gulono-1,4-lactone oxidase), PGI (Phosphoglucose isomerase), PMI (Phosphomannose isomerase), PMM (Phosphomannose mutase), GMP (GDP-D-mannose-pyrophosphorylase), GME (GDP-D-mannose epimerase), GGP (GDP-L-galactose-phosphorylase), GPP (L-galactose-1-phosphate phosphatase), GalDH (L-galactose dehydrogenase), GLDH (L-galactono-1,4-lactone dehydrogenase), MIOX (Myo-inositol oxygenase), GlcUR(Glucuronate reductase), AL (Aldonolactonase).

GENE INVOLVED IN PLANT VITAMIN C BIOSYNTHETIC PATHWAYS

The major ascorbic acid biosynthetic pathway involved in plants is the L-galactose/Smirnoff-Wheeler pathway. This 10-step metabolic process starts from D-glucose transformation into L-galactose in 8 metabolic steps and further ends with the conversion of L-galactose into L-ascorbic acid in 2 steps. Enzymes involved in the Smirnoff-Wheeler pathway are Hexokinase, Phosphogluco Isomerase, Phosphomannose Isomerase, Phosphomannose Mutase, GDP-D-Mannose Pyrophosphorylase, GDP-D-Mannose-3′,5′-Epimerase, GDP-L-Galactose Phosphorylase, L-Galactose-1-Phosphate Phosphatase, L-Galactose Dehydrogenase, and L-Galactono-1,4-Lactone Dehydrogenase. Except for mitochondrial enzyme L-Galactono-1,4-Lactone Dehydrogenase, all mentioned enzymes are localized in the cytosol.

Table 1 includes basic information on major enzymes involved in different ascorbate biosynthetic pathways in a plant; data is collected from the KEGG database. The following section of this review sheds some light on cloning techniques and the application of cloned genes of three enzymes of the Smirnoff-Wheeler pathway, i.e., GGP, GalDH, and GLDH. GGP catalyzes the first dedicated pathway step, i.e., conversion of GDP-L-galactose to L-galactose-1-phosphate; GalDH and GLDH are the terminal enzymes directly involved in ascorbate accumulation. These enzymes are selected for review as they perform irreversible conversion of the intermediate substrate, which may or may not be rate-limiting reactions. Overexpression of respective genes has shown increased ascorbate production, and suppression of these genes has shown decreased ascorbate accumulation. Advancements in cloning strategies of plant GGP, GalDH, and GLDH are summarized here:

Table 1: Enzymes and corresponding genes involved in ascorbate biosynthesis in *A. thaliana* and *C. annuum*.

Enzyme	*Plant Sp.*	*Gene*	*Chromosomal Location*	*Function (localization)*
Phosphomannose Isomerase KEGG ENZYME- 5.3.1.8	*Arabidopsis thaliana (thale cress)*	DIN9 Locus Tag- AT1G67070	1 (25041833..25044659)	Isomerize D-fructose-6-phosphate into D-mannose-6-phosphate (cytoplasmic enzyme)
	Capsicum annuum	LOC107858573	2 (69674155..69688215)	
Phosphomannose Mutase KEGG ENZYME- 5.4.2.8	*Arabidopsis thaliana (thale cress)*	PMM Locus Tag- AT2G45790	2 (18855675..18858018)	Coverts D-mannose-6-phosphate into D-mannose-1-phosphate (cytoplasmic enzyme)
	Capsicum annuum	LOC107839408	8 (126823231..126831135)	
GDP-D-Mannose Pyrophosphorylase KEGG ENZYME- 2.7.7.13	*Arabidopsis thaliana (thale cress)*	AT1G74910 Locus Tag- AT1G74910	1 (28135361..28138971)	Transforms D-mannose-1-phosphate to form GDP-D-mannose (cytoplasmic enzyme)
	Capsicum annuum	LOC107842837	9 (196165331..196170357)	
GDP-D-Mannose-3′,5′-Epimerase KEGG ENZYME- 5.1.3.18	*Arabidopsis thaliana (thale cress)*	GME Locus Tag- AT5G28840	5 (10862016..10864946)	Epimerize GDP-D-mannose to form GDP-L-galactose (cytoplasmic enzyme)
	Capsicum annuum	LOC107839422	8 (127108450..127111846)	
GDP-L-Galactose Phosphorylase KEGG ENZYME- 2.7.7.69	*Arabidopsis thaliana (thale cress)*	VTC2, VTC5 Locus tag- AT4G26850,AT5G55120	4 (13498956..13501701)	Catalyze the conversion of GDP-L-galactose to L-galactose-1-phosphate (cytoplasmic enzyme)
	Capsicum annuum	LOC107860116	2 (151884106..151890029)	
L-Galactose-1-Phosphate Phosphatase KEGG ENZYME- 3.1.3.93	*Arabidopsis thaliana (thale cress)*	VTC4 Locus tag- AT3G02870	3 (627512..629925)	Converts L-galactose-1-phosphate into L-galactose (cytoplasmic enzyme)
	Capsicum annuum	LOC107851391	12 (229242383..229247409)	
L-Galactose Dehydrogenase KEGG ENZYME- 1.1.1.316	*Arabidopsis thaliana (thale cress)*	AT4G33670 Locus tag- AT4G33670	4 (16169523..16171631)	Oxidizes L-galactose into L-galactono-1,4-lactone (cytoplasmic enzyme)
	Capsicum annuum	LOC107840121	8 (141830753..141834016)	
L-Galactono-1,4-Lactone Dehydrogenase KEGG ENZYME- 1.3.2.3	*Arabidopsis thaliana (thale cress)*	GLDH Locus tag- AT3G47930	3 (17684322..17687661)	Catalyze the conversion of L-galactono-1,4-lactone into L-ascorbic acid (mitochondrial enzyme)
	Capsicum annuum	LOC107843896	10 (202491623..202499290)	
L-gulonolactone oxidase KEGG ENZYME- 1.1.3.8	*Arabidopsis thaliana (thale cress)*	GulLO1 Locus tag- AT1G32300	1 (11651327..11653380)	Oxidizes L-gulono-1,4-lactone to L-ascorbate (membrane)
	Capsicum annuum	LOC107842223	9 (231054342..231055191)	
Myo-inositol oxygenase KEGG ENZYME- 1.13.99.1	*Arabidopsis thaliana (thale cress)*	MIOX1 Locus Tag- AT1G14520	1 (4968134..4970311)	Oxidizes Myo-inositol into D-glucuronic acid (cytoplasm)
	Capsicum annuum	LOC107840943	9 (6610724..6615895)	

GGP (GDP-L-Galactose Phosphorylase) (2.7.7.69). Genes (VTC1, VTC2, VTC3, VTC4) responsible for maintaining the vitamin C pool were identified using mutant Arabidopsis thaliana plants. Biochemical studies have revealed that VTC1 and VTC4 codes for GMP and GPP (Laing *et al.,* 2004; Conklin *et al.,* 2006; Conklin *et al.,* 1999). The activity of GGP, i.e., conversion of GDP-L-galactose to L-galactose-1-phosphate, was first demonstrated using cellular extract of pea seedlings. At pH 7.5, the enzyme activity was found to be higher. Thus the higher accumulation of L-galactose-1-phosphate was observed; the activity of the enzyme was observed to be highly stimulated in the presence of phosphate; the same set of experiments was also performed using *A. thaliana,* and similar results were obtained; however, the native molecular weight of GGP from pea extract and *A. thaliana* differ, i.e., 46kDA and 59kDa, respectively (AT4G26850, Dowdle *et al.,* 2004). In *A. thaliana,* VTC5 (AT5G55120), which is homologous to VTC2, also codes for GGP; However, VTC5 and VTC2 share many characteristics, but VTC2 is expressed significantly more and thus predominantly involved in Smirnoff-Wheeler pathway (Linster *et al.,* 2008). *A. thaliana* carrying mutated VTC2 showed decreased expression of GGP,which overall affects the ascorbate accumulation by 80%. In

contrast, knockout of VTC5 resulted in only a 10% decrease in ascorbate accumulation,suggesting the predominant role of VTC2 in ascorbate production via the Smirnoff-Wheeler pathway (Dowdle *et al.,* 2004).

The active site of GGP is at histidine, which is present at position 238; VTC2 and VTC5 contain HIT (histidine triad motif), which is primarily present in the superfamily of phosphorylases nucleotidyltransferase (Brenner, 2002). Linster *et al*. 2008 has shown that the primary function of the VTC2 gene product is to exhibit phosphorylase activity because of its GDP transferase activity. The result of overexpression of all the genes coding for enzymes of the Smirnoff-Wheeler pathway was checked and concluded that overexpression of the VTC2 gene resulted in a significant increase in ascorbate production. Instead, VTC2 is the only gene whose overexpression showed increased production of ascorbic acid (Yoshimura *et al.,* 2014; Ishikawa *et al.,* 2018).

To characterize the function of GGP, Linster *et al.,* 2007 PCR amplified VTC2 gene using a forward primer with sequence *5'-CACCATGTTGAAAATCAAAAGAG* and reverse primer with sequence *5'-TCACTGAAGGACAAGGCACTCGG,* which is specific for the coding region of AT4G26850. They cloned PCR product in vector Champion pET100/D-Topo, which gives protein product and N-terminal His-tag of 36 amino acids whose sequence is (MRGSHHHHHHGMASMTGGQQMGRDLYDDDD KDHPFT) and transformed it into E. coli BL21 Star (DE3). The transformation was followed by shaking incubation at 18°C, and protein overexpression was stimulated with isopropyl β-D-1-thio galactopyranoside; incubated cells were then harvested and sonicated. VTC2 gene product was separated from the lysate using a Ni2+ charged column, and imidazole was used for eluting bound protein. Further purification was done using an ultracentrifugal filter unit, and a Lowry assay was performed for concentration determination (Linster *et al*., 2007).

Enzymatic activity of GGP was assayed by incubating GDP-D-mannose with purified VTC2 gene product and P_i; only 2% of GDP-D-mannose was utilized in the reaction. Chromatographic analysis showed that incubation of GDP-D-mannose with GME resulted in the accumulation of GDP-D-mannose, GDP-L-galactose, and GDP-L-gulose in a ratio of 82:14:3 (Major *et al*., 2005). However, incubation of GME treated product with P_i and VTC gene product led to the accumulation of GDP-D-mannose, GDP-L-galactose, and GDP-L-gulose in a ratio of 97:1:2; this ensured that VTC2 only affects the concentration of GDP-L-galactose also the phosphorylase activity and P_i dependency of VTC2 product (Linster *et al.,* 2007).

Linster and coworkers found a second GGP coding gene in the A. thaliana genome, i.e., VTC5. Point mutated VTC2 carrying plant accumulates 30-50% of ascorbate compared to wild type, this raised query on the synthesis of ascorbate in VTC2 mutant A. thaliana. VTC2 homologous gene was identified using BLAST search; this new gene had locus tag AT5G55120. VTC5 has been cloned using a forward primer with sequence *5'-CACCATGTTGTTGAAGATCAAAAGAGTTCC-3'* and a reverse primer with sequence *5'-TCAATTAGAGACAGCCTCTTCTTTCACTG-3'*.

Expression was checked in vector Champion pET100/D-Topo and transformed in E. coli BL21 Star (DE3). The purification assay for separating VTC5 was the same as mentioned above for VTC2. VTC5 was found to have a 2.5-3.5 fold higher affinity for phosphate than P_i (Linster *et al.,* 2008).

Broad *et al.,* 2011 have cloned *Triticum aestivum*GGP using LR gateway cloning; GGP genes coding in *T. aestivum*is present on chromosome 4 and 5, three homologous genes code for GGP, which is present on subgenomes A, B, and D. They amplified *T. aestivum* GGP1 using forward primer with sequence *5'-GGCAATCTGTTAGGCAAGCA-3'* and reverse primer with sequence *5'-TGTCAAAAACAGGTATCAGCAATTT-3'*, the forward primer used for amplification of GGP2 have sequence *5'-AGTTCTTCCTAAATTCTCTCCTCCTT-3'* and reverse primer with sequence 5'-*TTGAGGAATCACCTCACCCTA-3'*. Amplified PCR products were purified by DNA Clean and Concentrator-5 (Zymo research product) and cloned into the pGEM-T vector (Broad *et al.,* 2019).

GalDH (L-galactose Dehydrogenase) (1.1.1.316). The second terminal enzyme involved in the Smirnoff-Wheeler pathway is GalDH which catalyzes the oxidation of L-galactose into L-galactono-1,4-lactone, the last known intermediate substrate for L-ascorbic acid biosynthesis. NAD^+ acts as an electron acceptor in the oxidation reaction of GalDH. Overall reaction catalyzed by GalDH is: *L-galactose* + NAD^+ = *L-galactono-1,4-lactone* + H^+ + *NADH*. Gatzek *et al.,* 2002 had purified GalDH for the first time from pea seedling extract; furthermore, they cloned and overexpressed the GalDH coding gene from *A. thaliana* in tobacco and established that 3.5 times increased expression of GalDH had no significant effect on ascorbate accumulation in tobacco leaf. However, to check the role of GalDH in ascorbate biosynthesis, they suppressed the gene coding for GalDH in *A. thaliana* and established that antisense suppression of GalDH had largely resulted in decreased ascorbate accumulation (Gatzek *et al.,* 2002).

GalDH is stable at pH 8.5-9 (Gatzek *et al.,* 2002); in *A. thaliana,*GalDH is coded by gene AT4G33670, and the enzyme has 319 amino acids, and the active site and binding site are present at 59th and 124th amino acid, respectively. The active site contains amino acid tyrosine which possesses proton donor activity. The molecular weight of purified GalDH from spinach is

36kDa, whereas *A. thaliana*GalDH is 42.4kDa (Mieda *et al.,* 2004; Gatzek *et al.,* 2002).

Gatzek *et al.,* (2002) have cloned the GalDH coding gene from *A. thaliana*. RNA was extracted from *A. thaliana* using the standard phenol-chloroform method, and further RNA was reverse transcribed using M-MLV reverse transcriptase. Resultant cDNA was served as a template in PCR reaction, and it was amplified with Taq polymerase using GalDH gene-specific forward and reverse primers whose sequences were *5'-TCACACATGACGAAAATAGAGCTTCG-3'* and *5'-CTTCTTTTAGTTCTGATGGATTCCACTTG-3'* respectively. Amplified DNA was purified using gel electrophoresis and a QIAquick gel extraction kit. Purified DNA was inserted into vector p-GEM-T and transformed into E. coli DH5α to increase gene content. The GalDH coding gene was digested from the pGem-T vector with *ApaI* and *PstI* treatment and subcloned into vector pBluescript. pBluescript was digested with *BamHI* and *EcoRI* to release the GalDH gene. GalDH gene was purified using gel electrophoresis and a QIAquick gel extraction kit at both steps. Finally,the GalDH gene was ligated to expression vector pRSETB digested with *BamHI* and *EcoRI*, and transformed in *E. coliBL21(DE3)lysS*. The recombinant GalDH expressed in pRSETB was purified using an affinity column containing HisBind Resin (Gatzek *et al.,* 2002).

Mieda *et al.,* 2004 have cloned spinach GalDH coding gene, in which RNA was extracted from spinach leaves and using oligodT primers, RNA was reverse transcribed through ReverTra Ace. Obtained cDNA was used as a template for PCR amplification by ExTaq DNA Polymerase using sense and antisense primers with sequences *5'-TAYGCNGARGGNTTYGAYTT-3'* and *5'-GCRTTDATNACNCCNACNCC-3'* respectively. To resolve the nucleotide sequence resultant amplified PCR product was cloned into vector pSTBlue-1.

After nucleotide sequencing of spinach GalDH, it was integrated into plasmid vector pGEX-5X-3. The GalDH was amplified using forward and reverse primers with sequence *5'-GAATTCCATGAACACCCATCAAAAATTAGA-3'* and *5'-AGAACACATCTAGTACCTGC-3'* and ExTaq Polymerase. The forward primer was attached with the restriction site of *EcoRI* for integration in the vector. Gel electrophoresis was used to purify amplified DNA fragments, and DNA fragments were integrated into vector pGEX-5X-3 and ultimately transformed into *E. coli BL21(DE3)* and allowed to grow under ampicillin selection pressure. As absorbance reached 0.6 at 600nm, the protein expression was stimulated with IPTG. Purification of recombinant protein was done with the help of GST-affinity chromatography (Mieda *et al.,* 2004).

Momma and Fujimoto, 2013 have cloned rice (*Oryza sativa*) GalDH to examine the expression and crystal structure using X-ray. They used cDNA (AK102223) as a template, and GalDH was PCR amplified using gene-specific forward and reverse primers with sequence *5'-ATGGAGCTCGCGAGCTCGGC-3'* and *5'-AGCTCTAGATCAGGCTTGCTCAATGCCACT-3'* respectively. Amplified fragments were cloned in expression vector pET-45b at *PmlI* and *AvrII* sites; before ligation, the amplified fragment was treated with *XbaI,*and the recombinant vector was transformed in *E. coli BL21(DE3)*. Here, protein expression was also induced with IPTG. The protein was purified using Ni2+-charged HiTrap chelating FF column chromatography and further purified by gel filtration column chromatography.

GLDH (L-Galactono-1,4-Lactone Dehydrogenase) (1.3.2.3). The last enzyme involved in the Smirnoff-Wheeler pathway is GLDH which catalyzes the oxidation of L-galactono-1,4-lactone to form the ultimate product, i.e., L-ascorbate acid. GLDH is a mitochondrial enzyme localized in intermembrane space that regulatesascorbic acid biosynthesis and facilitatesthe accretion of complex I. 1,4-benzoquinone and phenazine methosulfate act as an electron acceptor in the oxidation reaction of GLDH. Overall reaction catalyzed by GLDH is: *L-galactono-1,4-lactone + 4[Fe(III)cytochrome c] = L-ascorbate + 5 H+ + 4[Fe(II)cytochrome c]* (Pineau *et al.,* 2008). GLDH contains approximately 610 amino acids, and the transmembrane region is between 68-84 amino acids. GLDH is stable at ph 8.8 (Leferink *et al.,* 2008). Electrons generated by GLDH activity might get involved in the electron transport chain of mitochondrial complex III and IV (Bartoli *et al.,* 2000).

Pateraki *et al.,* 2004 have cloned GLDH from melon. They synthesized cDNA from extracted RNA using M-MuLV and oligo-dT primer. GLDH cDNA was then PCR amplified using degenerate forward and reverse primers with sequence *5'-A(CT)AT(ACT)CC(AGCT)TA(CT)AC(AGCT)GA(CT)(AG)C-3'* and *5'-CCA(AGCT)CC(AGCT)AC(AGCT)C(GT)(AG)TA(AGCT)CC(CT)TC-3'* respectively. Amplified PCR product was purified using gel electrophoresis followed by cloning in PGEM-T Easy vector and determining nucleotide sequence via LI-COR Long Reader 4200 automated sequencer. GLDH gene-specific primer was designed using nucleotide sequence. They cloned full-length GLDH cDNA in touch down PCR for upto 40 cycles using a gene-specific primer with initiation codon *5'-ATGCTCAACTTTCTCTCTCTTCGGCG-3'* and oligodT primers. Further, they checked the expression level of GLDH and found maximum expression in stems and petals (Pateraki *et al.,* 2004).

Landi *et al.,* 2015 have cloned the GLDH gene from lettuce using pre-designed primers (sequence not mentioned), which were responsible for cloning GLDH along with a *SalI* site. The amplified PCR product was purified with gel electrophoresis. After amplification, it

was integrated into the pGEM-T easy vector and then was further sub-cloned into competent *E. coli JM109.* Transcript/cDNA was integrated into pVDH282 using the *SalI* site and further transformed in *A. tumefaciens* (Landi *et al.,* 2015).

Rodriguez-Ruiz *et al.,* 2017 cloned the GLDH coding gene of pepper; they extracted total RNA content using the Trizol method and reverse transcribed it with AMV reverse transcriptase. Pepper GLDH gene was PCR amplified using forward and reverse primers with sequence *5'-TTACTCTTCAGAACTTTGC-3'* and *5'-GGATTGCATGTCACAACCAC-3'* respectively. Amplified products were detached and purified using gel electrophoresis and cloned into the pGME-T Easy vector.Furthermore, they analyzed the sequence and published it in NCBI (accession number: AY547352) (Rodriguez-Ruiz *et al.,* 2017).

An *et al.* (2007) have cloned GLDH from *Rosa roxburghii*; they isolated RNA by CTAB-LiCl method and utilized it for cDNA synthesis using RT-PCR with primers *5'-ATTTAGCTCGCTGTGGCCTCG-3'* and *5'-CTCAATCTTGGCCCAGTGTTC-3'*, desired amplified product was purified using gel electrophoresis and further cloned in vector pMD18-T and sequenced. Gene-specific primers were designed to rapidly amplify cDNA ends (RACE) based on the nucleotide sequence; 3' RACE was performed using primer *5'-CAATAGAGCAGCGCTGGACAG-3,'* and 5' RACE was performed using 5 sets of different primers. Further, they used this sequence for hybridization in Southern blotting (An *et al.,* 2007).

APPLICATION

Ascorbic acid plays a vital antioxidant role throughout the body; ascorbate can directly stabilize ROS by donating electrons because it contains C2 and C3,an enediol group. C3 hydroxyl group is deprotonated at physiological pH and forms a monovalent anion, whereas by donating one or two electrons from the enediol group, ascorbic acid forms monodehydroascorbate (MDHA) or dehydroascorbate (DHA) (Tripathi *et al.,* 2009; Paciolla *et al.,* 2016). Ascorbic acid also plays definite roles, such as preventing eyes from solar radiation oxidative damage; inhibiting the synthesis of carcinogenic molecules like nitrosamine and nitric oxide; cofactor for hydroxylase, vasopressin, etc. (Brubaker *et al.,* 2000; Huang *et al.,* 2000).

As ascorbate is widely involved in cell functioning and metabolism, attempts have been made to scale up ascorbate production utilizing different enzymes. Overexpressing different enzymes of the Smirnoff-Wheeler pathway,especially those responsible for rate-limiting reactions or irreversible reactions, may enhance the production of ascorbic acid and reveal the effect of the enzyme on the plant, such as resistance against stress, etc. Attempts to increase ascorbate concentration in different plant species by expressing gene coding for enzymes GGP, GalDH and GLDH have gained some success; ascorbate content has been increased 3-fold by transient expression of kiwifruit GGP in tobacco.However, the enzyme activity was enhanced upto 50 folds (Laing *et al.,* 2007). Overexpression of GGP of *Oryza sativa* under constitutive CaMV35 promoter has increased ascorbate concentration by 5-fold in germinated brown rice. But, non germinated brown rice showed no significant difference compared to wild type. Constitutive overexpression of GGP in rice was not related to salt tolerance. It was inconclusive that ascorbic acid is involved in iron availability (Broad *et al.,* 2020). Transgenic plant p571GGP that overexpress GGP coding gene resulted in increased ascorbic acid content; under salt stress conditions, p571GGP showed relatively larger plant height and root length, andthe transgenic plant was 67% more effective to resist membrane ion leakage as compared to wild type (Zhang *et al.,* 2015). Overexpression of the GGP coding gene also corresponds to an increased ascorbic acid-DHA ratio during chilling stress conditions and provides higher tolerance against chilling. Under chilling stress, transgenic tobacco accumulated less ROS (Wang *et al.,* 2014). Three GGP expressing (under 35S promoter) transgenic tomato lines resulted in 2.6-3.5 fold increased ascorbate concentration; however, it negatively affected fruit shape and yield of no or non-viable seeds. Polyphenolic content in transgenic tomatoes was doubled in plants, which showed increased ascorbate concentration, and there were no significant differences in transgenic carotenoid content compared to control (Bulley *et al.,* 2012).

Twenty transgenic tobacco lines expressing *A. thaliana*GalDH under CaMV35S promoter showed no significant difference butthe increased activity of GalDH by 3.5 fold. However, increased enzyme activity did not affect ascorbate concentration, butthe exogenous supply of L-galactose to the leave expressing 3.5 fold of GalDH showed a very slight increase in ascorbate concentration (Gatzek *et al.,* 2002). E. coli expressing recombinant GalDH cDNA of spinach resulted in an accumulation of 10% of the entire protein content. It was found that GalDH is susceptible to increased concentration of ascorbic acid (Mieda *et al.,* 2004).

At first, there was no conclusive evidence that overexpression of GLDH corresponds to enhanced production of ascorbic acid; overexpression of GLDH in wheat leaves was not shown any significant difference compared to control under drought stress (Bartoli *et al.,* 2005). BY-2 transgenic tobacco cells which overexpress GLDH under CaMV 35-S promoter showed higher activity of GLDH, which is positively correlated with enhanced production of ascorbic acid (Tokunaga *et al.,* 2005). To check the effect of GLDH

on rice plant growth, GLDH is suppressed and overexpressed; GLDH suppression led to a decrease in rice plant height, root and leaf weight (56.4, 18.2, and 16.5%, respectively) as compared to control wild type. However, overexpression of GLDH resulted in increased ascorbic acid content but had no direct effect on plant growth. Overexpression of GLDH resulted in increased seed sets in rice (Liu *et al.,* 2011). Out of 4 transgenic tobacco lines carrying *Rosa roxburghii* GLDH under CaMV35S promoter,two lines showed enhanced activity of GLDH and accumulation of ascorbic acid compared to wild type. GLDH overexpressing transgenic lines also showed tolerance against salt stress. Under salt stress, these transgenic lines grew with better shoot length and weight than wild type and resisted oxidative damage (Liu *et al.,* 2013). Overexpression of GLDH cDNA of lettuce showed 30% more accumulation of ascorbic acid, and the increased ascorbic acid concentration corresponds to reduced leaf browning (Landi *et al.,* 2015). The exclusive GLDH allele of *Triticum aestivum,* namely TaGLDH-A1b, has shown higher tolerance against drought (Zhang *et al.,* 2016).

FUTURE SCOPE

Advancement in the field of ascorbic acid biochemistry, including metabolism and biosynthesis, opens a new path for enhancing ascorbic acid content in plants and microbes. This also favored the cloning of genes involved in ascorbate biosynthesis, most of the genes are well explored, and many cloning strategies have been published inthe last two decades. Transgenic plants have been developed by cloning and expressing genes of different ascorbic acid biosynthetic pathways. These transgenic plants are made conducive to optimize ascorbic acid biosynthesis. Some model plants, such as *A. thaliana*, *Oryza sativa*, tobacco, etc., showed increased synthesis and accumulation of ascorbic acid by overexpression of genes of the ascorbate biosynthetic pathway. However, overexpression of a single gene can be supplementary rate-limiting and has restricted significance. It is concluded that enhanced enzyme activity may correlate with increased ascorbate biosynthesis/accumulation, but it is not unswervingly proportional. For further studies combination of two or more biosynthetic genes can be used in a single model plant to regulate ascorbate synthesis, and the effect of other metabolic products might be exploited. Till now, leaves have been in focus for expressing cloned genes. Therefore the impact of cloned genes could also be explored on other plant parts such as flowers, fruit, etc. An ideal protocol must be developed to transform the result observed in the model plant into crops that can be consumed.

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