

# Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant

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**The biosynthetic pathway of L-ascorbic acid (vitamin C) in plants has been established for several years. However, recent reports describe alternative pathways, revealing a more complex picture of L-ascorbic acid biosynthesis than had been expected. GDP-L-gulose and *myo*-inositol are proposed as new intermediates in L-ascorbic acid biosynthesis, indicating that part of the animal pathway might also be operating in plants. Enzymatic studies on the GDP-mannose-3',5'-epimerase and L-galactono-1,4-lactone dehydrogenase suggest that they are important regulatory steps for L-ascorbic acid biosynthesis.**

L-ascorbic acid is the trivial name for the six-carbon sugar derivative L-threo-hex-2-enono-1,4-lactone. The biosynthesis of L-ascorbic acid from D-glucose was first reported in rats [1]. L-ascorbic acid can be synthesized *de novo* in the hexuronic acid pathway of the liver or the kidney of species having L-gulonolactone oxidase activity (Figure 1, pink background). Radiotracer studies showed that this pathway involves an inversion of configuration. However, humans, non-human primates and a few other mammals cannot synthesize L-ascorbic acid because the gene encoding the L-gulonolactone oxidase, the enzyme catalysing the last step in the biosynthesis, is highly mutated and non-functional [2]. Because of the important antioxidant and metabolic functions of this compound it must therefore be incorporated as part of the human diet, and is known as 'vitamin C'. L-ascorbic acid in plants, the main source of vitamin C for humans, is also an essential compound for plants, with important roles as an antioxidant and as a modulator of plant development through hormone signalling [3]. Accordingly, its synthesis and steady-state level in different cell compartments must be tightly regulated. Early experiments showed that in detached strawberry fruits, asymmetrically labelled D-glucose was converted to L-ascorbic acid over a pathway without inversion of the carbon chain [4]. It was also known that L-galactono-1,4-lactone was a precursor of L-ascorbic acid in cress (*Lepidium sativum*) seedlings [5]. Moreover, this compound was enzymatically converted to L-ascorbic acid by mitochondria from pea and mung bean (*Vicia radiata*) seedlings [6]. Based on biochemical

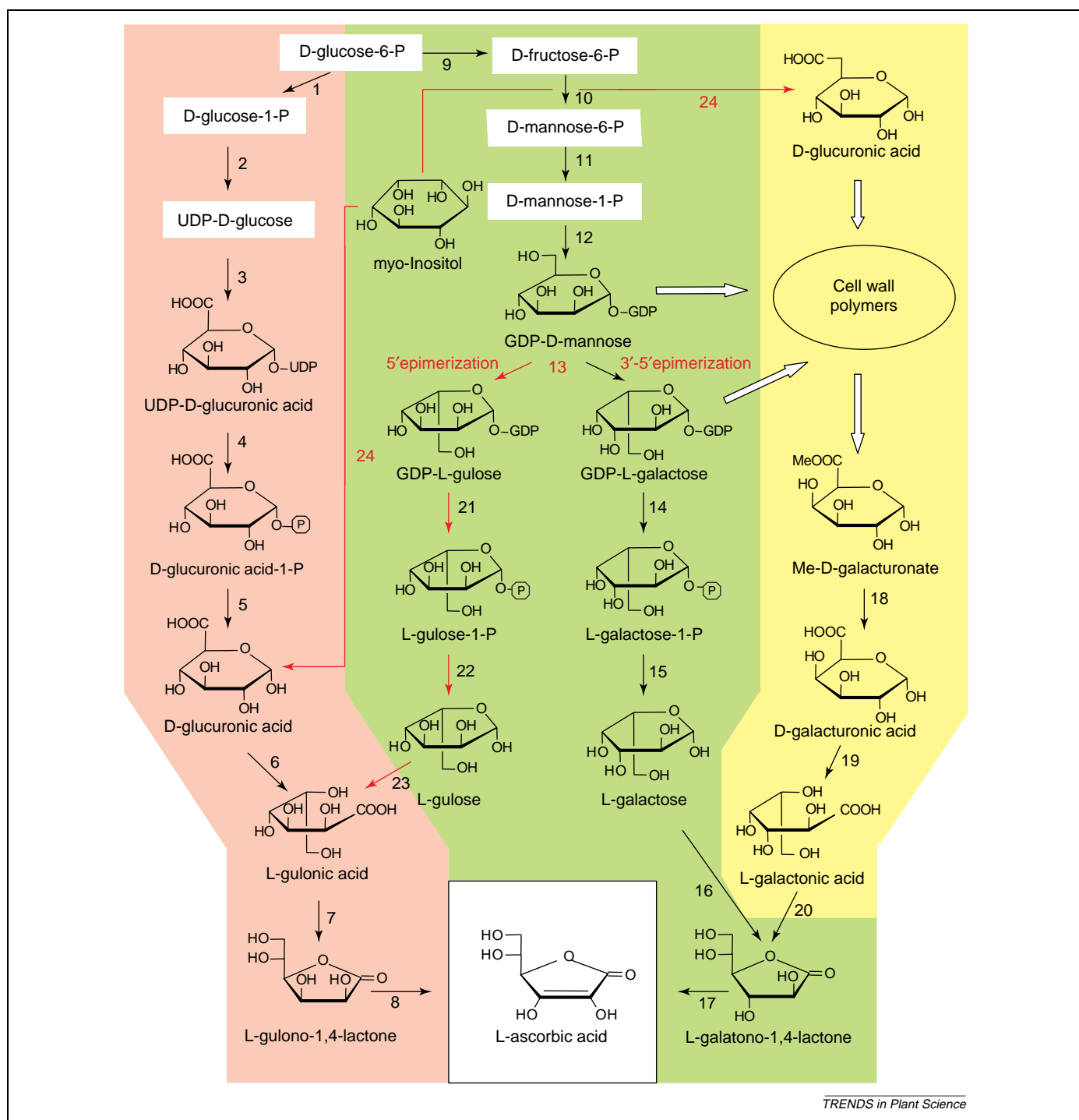
evidence, in 1998 a pathway was proposed for L-ascorbic acid in plants that combined the two facts: the non-inversion of the carbon skeleton and the occurrence of L-galactono-1,4-lactone as the immediate precursor of L-ascorbic acid [7] (Figure 1). Since then, molecular and genetic evidence demonstrating the function of this pathway in photosynthetic tissues has been reported [8,9]. This pathway involves the conversion of GDP-D-mannose to GDP-L-galactose catalysed by a GDP-mannose-3',5'-epimerase [10]. L-galactose released from the nucleotide is the immediate precursor of L-galactono-1,4-lactone, which by action of a dehydrogenase is converted to L-ascorbic acid (Figure 1, green background). Feeding experiments using precursors have shown that the methyl ester of D-galacturonic acid causes a significant increase in the L-ascorbic acid content of cress seedlings and *Arabidopsis* cultured cells [5,11]. It is also known that D-galacturonic acid-1-<sup>14</sup>C is metabolized to L-ascorbic acid-6-<sup>14</sup>C by an inversion pathway in detached ripening strawberry fruit [12]. A uronic acid pathway that accommodates both findings, the role played by methyl ester of D-galacturonic acid as precursor and the occurrence of an inversion pathway in some plant tissues, was previously proposed [13]. Molecular evidence of this pathway came with the cloning and characterization of a D-galacturonic acid reductase from strawberry fruits [14]. In this pathway, pectin-derived D-galacturonic acid is reduced to L-galactonic acid, which in turn is spontaneously converted to L-galactono-1,4 lactone. This compound is the substrate of the L-galactono-1,4-lactone dehydrogenase enzyme (Figure 1, yellow background).

## Intrigue of an equilibrium constant

The enzyme GDP-mannose 3',5'-epimerase was known to catalyse the conversion of GDP-D-mannose to GDP-L-galactose [10]. However, abnormal values in the apparent equilibrium constant reached for this interconversion catalysed by both the recombinant and the native enzyme led the authors to formulate the hypothesis that GDP-L-galactose was not the only epimerization product [15]. The new compound was identified as GDP-L-gulose, which results from the 5'-epimerization of the GDP-D-mannose. Therefore, the GDP-mannose-3',5'-epimerase enzyme catalyses two distinct epimerization reactions that produce either GDP-L-galactose or GDP-L-gulose. The type of epimerization seems to be

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Available online 2 November 2004



TRENDS in Plant Science

**Figure 1.** Proposed biosynthetic pathways of L-ascorbic acid in animals (reactions 1–8) and plants (reactions 9–24). Red arrows indicate enzymatic activities of the new pathways leading to L-ascorbic acid biosynthesis [15,19]. Enzymes catalyzing the numbered reactions are: 1, phosphoglucomutase; 2, UDP-glucose pyrophosphorylase; 3, UDP-glucose dehydrogenase; 4, glucuronate-1-phosphate uridylyltransferase; 5, glucurono kinase; 6, glucuronate reductase; 7, aldono-lactonase; 8, gulono-1,4-lactone dehydrogenase; 9, glucose-6-phosphate isomerase; 10, mannose-6-phosphate isomerase; 11, phosphomannomutase; 12, GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase); 13, GDP-mannose-3',5'-epimerase; 14, phosphodiesterase; 15, sugar phosphatase; 16, L-galactose dehydrogenase; 17, L-galactono-1,4-lactone dehydrogenase; 18, methyltransferase; 19, D-galacturonate reductase; 20, aldono-lactonase; 21, phosphodiesterase; 22, sugar phosphatase; 23, L-gulose dehydrogenase; 24, myo-Inositol oxygenase.

dependant on the molecular form of the enzyme [15]. This reaction product established a new branch of the pathway in plants and a link with the animal pathway (Figure 1). The following steps in the branch have not been described in plants, but the intermediate L-gulonic acid [16] and L-gulono-1,4-lactone dehydrogenase activity [15] have been reported.

### Myo-inositol: a new player in the game?

The functionality in plants of a pathway similar to the one described in animals has never been discarded. Over-expression in lettuce and tobacco of the rat gene encoding the L-gulono-1,4-lactone oxidase increased the L-ascorbic acid level of the transgenic plants by up to sevenfold [17]. More recently, it has been shown that ectopic expression of

this gene rescued leaf L-ascorbic acid content in all five *Arabidopsis* (*vtc*) mutants that were deficient in L-ascorbic acid [18]. This means that either the rat enzyme is using L-galactono-1,4-lactone as a substrate or that the genuine substrate, L-gulonono-1,4-lactone, is being produced in *Arabidopsis*. Because the production of L-galactono-1,4-lactone is reduced in the *vtc1* mutant, the second possibility seems more likely. This would indicate that an alternative pathway is present in plants that can circumvent some steps of the D-mannose pathway. However, these results must be carefully analysed because ectopic overexpression of a new gene could introduce a novel pathway and does not necessarily indicate that the pathway operates in wild-type plants. It was also known that an early intermediate of the animal pathway, D-glucuronic acid, can be produced by the action of a *myo*-inositol oxygenase, but this possibility had not been assayed before in plants. The availability of the complete genome sequence for *Arabidopsis* allowed researchers to search for plant homologues of the *myo*-inositol oxygenase gene from pig [19]. Four open reading frames with homology to this gene at chromosomes 1, 2, 4 and 5 were found. Further refinement of the search in protein documentation resources pointed to a coding region from the gene present at chromosome 4. After being cloned and expressed in bacteria, the recombinant protein showed *myo*-inositol oxygenase activity. The ectopic expression of the *myo*-inositol oxygenase gene significantly increased L-ascorbic acid levels in transgenic *Arabidopsis* [19], but the contribution of *myo*-inositol in L-ascorbic acid biosynthesis *in vivo* has yet to be determined. Tracer studies with detached ripening strawberry fruits and parsley leaves appeared to rule out *myo*-inositol as an important precursor of L-ascorbic acid in these tissues [20].

All recent publications seem to indicate that multiple L-ascorbic acid biosynthetic pathways are functioning in plants (Figure 1). However, definitive proof of their occurrence must be obtained either by using quantitative radiolabelling studies or by reverse genetics (i.e. knocking out the genes encoding the enzymes that are hypothesized to be involved). The last possibility might be complicated by the presence of gene families, as occurs with the gene encoding the *myo*-inositol oxygenase [19].

### Regulation is being disclosed

L-ascorbic acid is present in plant species in concentrations that range from an estimated 300 mM in the chloroplast stroma to less than 20 mM in other plant tissues [21]. In spite of this high and wide-ranged value, the multiple roles played by this molecule in crucial physiological processes mean that the steady-state values are tightly controlled [21]. This is achieved at various levels, such as enzyme activity, gene expression in response to developmental and environmental cues, regeneration of the oxidized form, compartmentation and transport of the molecule. Regarding enzyme activities, feedback inhibition of L-ascorbic acid biosynthesis has been known for some time [22]. The GDP-mannose-3',5'-epimerase from *Arabidopsis* is a good candidate for being an important regulator in L-ascorbic acid levels. The partially purified *Arabidopsis* enzyme is inhibited *in vitro*

by the final products L-ascorbic acid and L-galactono-1,4-lactone [15]. The observation that it precedes the committed step in the irreversible hydrolysis of the highly energetic glycosyl-pyrophosphoryl linkage supports the idea of a key control point in the biosynthesis pathway (Figure 1). It is interesting that the stimulation of enzyme activity by the oxidized nicotinamide nucleotides (NAD<sup>+</sup> and NADP<sup>+</sup>) and the inhibition by their reduced forms (NADH and NADPH) associates the enzyme activity with the redox state of the cell [15]. Another recent report also supports this linkage between L-ascorbic acid biosynthesis and the cell redox state [23]. It was found that electron flow through the mitochondrial complex I is required for the optimal rate of L-ascorbic acid biosynthesis [23]. This study reveals a physical link between the L-galactono-1,4-lactone dehydrogenase (GLDH) enzyme and the mitochondrial complex I but the mechanism of this interaction remains to be established. It has been known for some time that GLDH, the last enzyme in the plant-specific pathway, is an integral protein of the inner mitochondrial membrane [24]. The L-ascorbic acid level of plant cells is sensitive to stress conditions such as exposure to ozone and pathogen attack [25]. The GDP-mannose 3',5'-epimerase can be co-purified with a heat shock protein [15], suggesting that this interaction is part of the plant regulation mechanism of this enzyme under stress. Albeit attractive, this hypothesis needs further investigation.

There are only a few reports on the transcriptional regulation of genes related to L-ascorbic acid biosynthesis. Two main aspects have been analysed: the feedback inhibition by L-ascorbic acid and the effect of light. In tobacco suspension cells, exogenous addition of L-ascorbic acid results in a marked decrease of transcripts of genes encoding the GDP-mannose pyrophosphorylase and L-galactono-1,4-lactone dehydrogenase (Figure 1) [26]. Relative to the effect of light, in tobacco leaves there is a marked decrease of transcripts for the genes encoding GDP-mannose pyrophosphorylase and L-galactono-1,4-lactone dehydrogenase in non-irradiated halves of the tobacco leaf [26]. Expression at the transcriptional level of this gene correlates with diurnal changes of L-ascorbic acid in *Arabidopsis* [27]. Whether or not the expression of this gene determines the content of L-ascorbic acid has yet to be established.

L-ascorbic acid is a major contributor to the redox state of the cell, therefore processes that oxidize this compound should also be considered. Recent studies on the regeneration of the reduced form of L-ascorbic acid have shown that increasing the expression of the dehydroascorbate reductase gene not only increased the reduced:oxidized ratio but also produced a net increase in the content of L-ascorbic acid [28]. This effect might result from enhanced rescue of dehydroascorbic acid, which undergoes irreversible hydrolysis to 2,3-diketogulonic acid [29].

### Specific pathways for different cell types

Fluxes in the network depicted in Figure 1 are dependent on cell specialization and metabolic regulation. It is known that L-ascorbic acid content in plants changes with light [26], hour of the day [27,30], age [31], plant tissue [19] and

cell compartment [32]. Theoretically, all these changes should be explained by the functioning of a complete metabolic network of L-ascorbic acid biosynthesis, catabolism and recycling. For example, D-galacturonate reductase activity has been reported in ripening fruit tissue of strawberries [14]. It is likely that the flux through this branch is dependent upon the availability of the substrate D-galacturonate. This compound appears as a product of the turnover of cell wall pectins in senescing cells. The gene encoding the *myo*-inositol oxygenase enzyme is primarily expressed in flowers and leaves of *Arabidopsis* [19]. The reaction product, D-glucuronic acid, can follow two pathways in plant cells, either the biosynthesis of L-ascorbic acid or the supply of precursors for the synthesis of cell wall polymers such as pectin and hemicellulose [33]. It is clear that green tissues can favour another pathway because of light activation and the requirement for large carbon pools. The scenario can be specific. Thus, it has been recently reported that the level of L-ascorbic acid in guard cells changes in tobacco leaves depending on whether they are collected in the morning or in the afternoon [30]. Recycling from dehydroascorbic acid is certainly involved in these diurnal changes [30], but it is a challenge to determine whether or not any of the biosynthetic pathways are involved in this redox balance.

Studies on L-ascorbic acid transport are crucial to associate the different pathways to specific plant organs and/or developmental stages. For example, transport of L-ascorbic acid from source leaf to root tips, shoots and floral organs has recently been reported [34]. Phloem translocation appears to be crucial for the supply of L-ascorbic acid to sink organs. In addition to long-distance transport, L-ascorbic acid translocation through different cell membranes is relevant to understanding the interplay between the biosynthetic pathways, but information in this area is still limited [34,35].

### Black hole of catabolism

*In vivo* levels of L-ascorbic acid result from the balance between synthesis and degradation. This is important because there is some evidence of a relatively high turnover rate of this compound in some plant tissues as well as suggestions that the turnover rate might contribute to controlling L-ascorbic acid content [22]. The first catabolic step is oxidation, which produces successively the monodehydroascorbate radical and dehydroascorbate. Dehydroascorbate undergoes irreversible hydrolysis to 2,3-diketo-L-gulonic acid, if non-enzymatically recycled to L-ascorbic acid. The various recycling pathways to L-ascorbic acid are known and their contribution to maintaining the L-ascorbic acid level has been well documented [28,30]. For example, overexpression of a wheat dehydroascorbate reductase in tobacco and maize increased not only the ratio of the reduced to the oxidized form but also the level of L-ascorbic acid by 2–4-fold [28]. The best explanation of this net increase in L-ascorbic acid is that dehydroascorbic acid is being recycled to L-ascorbic acid before being converted to 2,3-diketo-L-gulonic acid. Metabolites downstream from 2,3-diketo-L-gulonic acid in the catabolic pathway are still unknown. Different products from L-ascorbic acid catabolism have been reported

to accumulate as end products, such as L-threonic acid, L-tartaric acid, glyceric acid and oxalic acid [1]. It is recognized that recycling through triose and hexose phosphates can occur [1], but still this information remains elusive.

For many years it has been known that the antioxidant role played by L-ascorbic acid is what makes this small molecule a crucial component of the plant response to different stress agents [25]. More recently, it has been involved in some cell-specific events such as stomatal closure [30]. Moreover, the multiple analysis of gene expression in *Arabidopsis* mutants deficient in L-ascorbic acid has revealed that this compound can affect the expression of hundreds of genes [3]. All these findings point to L-ascorbic acid being a signal molecule that is involved in many developmental and defense responses in plants. The corollary is that cell and compartment concentration of L-ascorbic acid must be tightly regulated. The steady-state level of L-ascorbic acid in plants must be the result of its synthesis, degradation and transport. The reports highlighted here have significantly improved our knowledge of the biosynthetic pathways and its regulation. Some players in this game have come to light in the past few years and have been assembled in the biosynthetic network (Figure 1). Degradation and transport have not been discussed, partly because the information is still limited. The next challenge will be to determine which branch (or new ones) of the network functions under specific developmental or environmental conditions, in other words, the versatility and plasticity of the network.

### Acknowledgements

We thank José R. Botella for critically reading the manuscript. This work was supported by grant BIO2001–1958-C04–01 from the Ministry of Education and Science (Spain).

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