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Molecules of Interest

The VTC2 cycle and the *de novo* biosynthesis pathways for vitamin C in plants: An opinion

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Abstract

The recent identification of the VTC2 enzyme (GDP-L-galactose: hexose 1-phosphate guanylyltransferase) that forms with the GDP-mannose 3″,5″ epimerase an energy-conserving hub for the production of GDP-hexoses and L-galactose 1-phosphate [Laing et al., Proc. Natl. Acad. Sci. USA 104, 2007, 9534–9539], is a major breakthrough in our understanding of the biosynthesis of L-ascorbic acid (vitamin C) in plants. The observation that the VTC2 enzyme can use glucose 1-phosphate and GDP-D-glucose as substrates, and the long-known existence of an enigmatic GDP-D-mannose 2″-epimerase activity, have led us to the proposal of an extended VTC2 cycle that links photosynthesis with the biosynthesis of vitamin C and the cell-wall metabolism in plants. An evolutionary scenario is discussed for the acquisition of genes of eubacterial origin for the *de novo* synthesis of L-ascorbic acid in green algae and plants.

Keywords: *Arabidopsis thaliana*; Biosynthesis; *Chlamydomonas reinhardtii*; L-Ascorbic acid; Pathway; Review

1. Introduction

Since the discovery of ascorbic acid by Szent-Györgyi in 1928, followed by the structural determination (in 1933) and chemical synthesis from L-xylosone (in 1933) and D-glucose (in 1934) (Davies et al., 1991), vitamin C and its biosynthesis in plants and animals has aroused a lot of interest and passion among the scientists all over the world.

Development of transgenic plant technology has found applications in the field of micronutrients and biofortification, and significant success has been achieved in generating transgenic plants with increased levels of vitamin E (α-tocopherol) (Shintani and DellaPenna, 1998), provitamin A (β-caroten) (Ye et al., 2000), and folic acid (vitamin B_9) (Diaz de la Garza et al., 2007). However, despite almost two decades of efforts and an unquestionable research progress, similar success could not be achieved in the field of vitamin C biosynthesis. Quite deceivingly, genetically modified plants overexpressing plant enzymes for vitamin C synthesis contained only 2–3 higher ascorbic acid levels (Agius et al., 2003; Lorence et al., 2004). Surprisingly, the highest increase of vitamin C content (5–7 folds) was reported in plants overexpressing the rat gene for L-ascorbic acid synthesis, L-gulono-1,4-lactone oxidase (Jain and Nessler, 2000). Perhaps, using other non-plant genes to generate plants with higher vitamin C contents as, for example, bacterial L-gulono-1,4-lactone dehydrogenases (Wolucka and Communi, 2006), could be an option.

Plants contain variable amounts of L-ascorbic acid that may range from low micromolar to 0.3 molar concentrations, depending on the plant species, organ, tissue, cell, and the environmental conditions. The variability of ascorbate contents is related to specific functions of vitamin C in plants and, probably, reflects the activity of different biosynthetic but also catabolic pathways in a given plant cell or even cell compartment.

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2. De novo biosynthesis of L-ascorbic acid in plants

Several routes for plant vitamin C synthesis have been proposed and can be roughly classified as the *de novo* (non-inversion) and salvage (mainly inversion) pathways (Loewus, 1963). Whereas the salvage pathways have been evoked a half century ago (Isherwood et al., 1953; Loewus, 1963), the partially deduced *de novo* D-mannose route proposed by Wheeler et al. (1998) was new and based, essentially, on the discovery of the plant L-galactose dehydrogenase (Smirnoff and Wheeler, 1999) (Fig. 1). In the meantime, vitamin C-deficient (*vtc*) mutants of *Arabidopsis thaliana* were generated that have 30–50% of the wild type ascorbic acid level and bear a point mutation in some of the genes of the D-mannose route (*vtc1* and *vtc4*) but also in genes of poorly understood function (*vtc2* and *vtc3*) (Conklin et al., 1996, 2000). The majority of the proposed enzymes and the corresponding genes were identified, including: phosphomannose mutase (Qian et al., 2007), GDP-D-mannose pyrophosphorylase (VTC1) (Conklin et al., 1999), GDP-D-mannose 3″,5″-epimerase (Wolucka et al., 2001), L-galactose/*myo*-inositol 1-phosphate phosphatase (VTC4) (Laing et al., 2004; Conklin et al., 2006), L-galactose dehydrogenase (Gatzek et al., 2002), L-galactono-1,4-lactone dehydrogenase (Ostergaard et al., 1997). Only very recently, two apparently contradictory reports regarding the enzymatic activity of the VTC2 gene product have been published (Laing et al., 2007; Linster et al., 2007) (see below). The vitamin C-related enzymes, except the VTC1 GDP-mannose pyrophosphorylase, have been, at least partially, characterized, and the crystal structure of the first enzyme for vitamin C, the GDP-mannose 3″,5″-epimerase, was determined (Major et al., 2005). All of the identified until now *vtc* mutations affect proteins that are also involved in the cell-wall/glycoprotein synthesis (*vtc1*, *vtc2*, *vtc4*). Significantly, mutants with a lesion in enzymes that catalyze one of the last steps of ascorbate synthesis could not be isolated. This observation suggests either that such mutations are lethal, or, more probably, that enzymes catalyzing the last steps of vitamin C synthesis are redundant and can compensate for each other in plants (see below).

However, a few unexpected experimental facts have challenged the D-mannose route of Wheeler et al. (1998). Firstly, D-mannose 6-phosphate isomerase, a key enzyme of the D-mannose pathway that links D-mannose metabolism with common D-glucose/fructose intermediates of general metabolism, is often not expressed in plants (Gao et al., 2005; Zhu et al., 2005), unless in dark (Fujiki et al., 2001). Paradoxically, D-mannose 6-phosphate isomerase is used as an antibiotic-free positive selection marker in plant biotechnology (Schiermeier, 2000; Privalle, 2002; Wenck and Hansen, 2005). At the absence of the phosphomannose isomerase activity, the origin of D-mannose 6-phosphate in the L-ascorbic acid route proposed by Wheeler et al. (1998) (Fig. 1) is unclear.

Secondly, the highly conserved among both dicotyledonous and monocotyledonous plants (about 90% identity) GDP-D-mannose 3″,5″-epimerase forms a previously unsuspected, novel product: GDP-L-gulose (Wolucka and Van Montagu, 2003; Major et al., 2005; Watanabe et al., 2006), in addition to the well-known GDP-L-galactose. The GDP-mannose 3″,5″-epimerase of green algae (74% identity with the *Arabidopsis* epimerase) has, probably, a similar activity because, in contrast to higher plants that do not produce gulose-containing polymers, gulose is a structural component of certain green algae (Mengele and Sumper, 1992). It is hard to believe that a useless side activity of the epimerase would be so conserved in higher plants, in particular because both L-gulose and L-gulono-1,4-lactone serve as precursors of L-ascorbic acid in plant cells (Wolucka and Van Montagu, 2003).

Thirdly, the highly specific plant L-galactono-1,4-lactone dehydrogenase that was used as an argument for the absolute requirement of L-galactono-1,4-lactone substrate and its L-galactose precursor (Wheeler et al., 1998), is not the only enzyme for the last step of vitamin C synthesis in plants. The L-gulono-1,4-lactone dehydrogenase activity is present in plant cell extracts, and six putative L-gulono-1,4-lactone dehydrogenase genes (At2g46740, At2g46750,

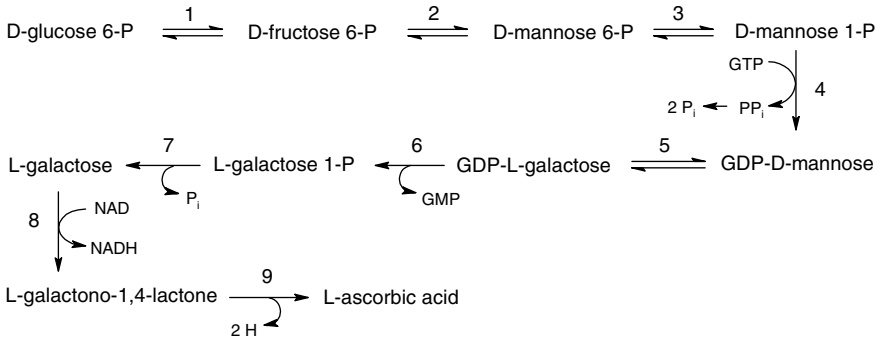

Fig. 1. The originally proposed D-mannose pathway for L-ascorbic acid (vitamin C) in plants. (Wheeler et al., 1998). Enzymes: 1, phosphoglucose isomerase; 2, phosphomannose isomerase; 3, phosphomannose mutase; 4, GDP-D-mannose pyrophosphorylase; 5, GDP-D-mannose 3″,5″-epimerase; 6, GDP-L-galactose phosphodiesterase; 7, L-galactose 1-P phosphatase; 8, L-galactose dehydrogenase; 9, L-galactono-1,4-lactone dehydrogenase. Note that phosphomannose isomerase (reaction 2) is often not detectable in plants (see the text).

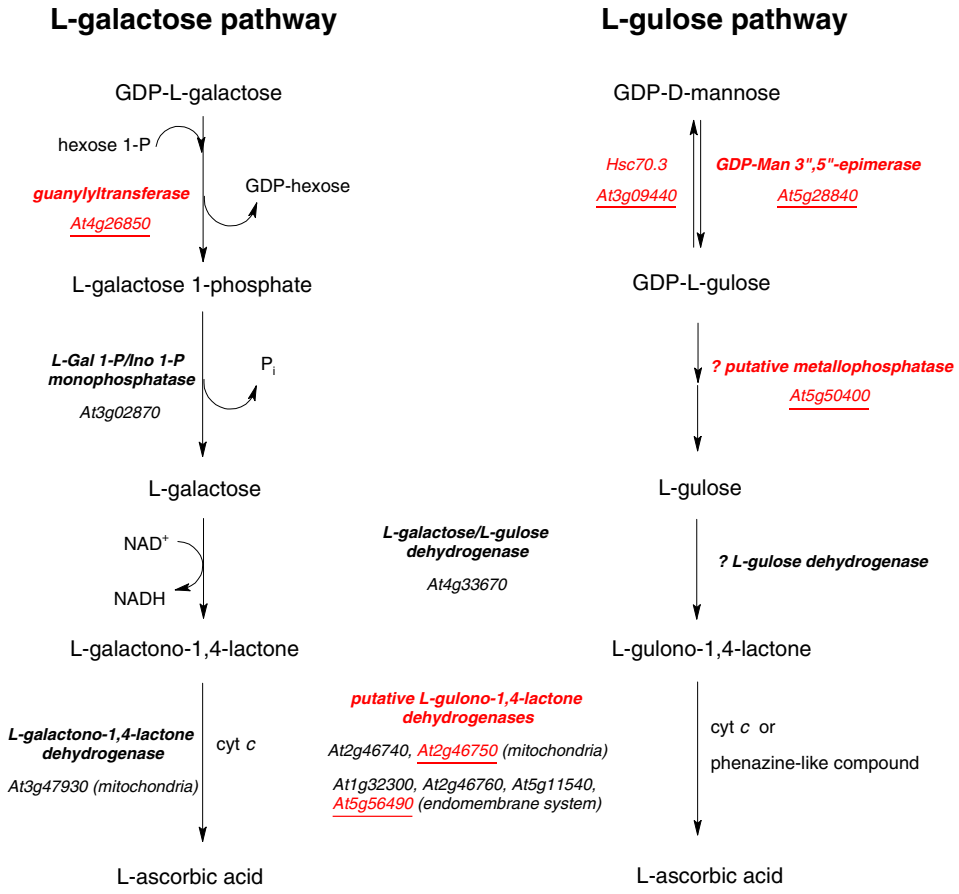

Fig. 2. The L-galactose and L-gulose pathways for the *de novo* synthesis of L-ascorbic acid (vitamin C) in plants. The first specific step of the L-galactose pathway (Wheeler et al., 1998) is the formation of L-galactose 1-phosphate from the GDP-L-galactose and hexose 1-phosphate substrates catalyzed by the GDP-L-galactose/GDP-D-glucose : hexose 1-P guanylyltransferase (VTC2) (Jander et al., 2002; Laing et al., 2007), whereas the last step is catalyzed by the highly specific, mitochondrial L-galactono-1,4-lactone dehydrogenase. The first specific step of the L-gulose pathway (Wolucka and Van Montagu, 2003) is a reversible conversion of GDP-D-mannose to GDP-L-gulose catalyzed by the GDP-D-mannose 3″,5″-epimerase, and the last step is the oxidation of L-gulono-1,4-lactone to L-ascorbic acid catalyzed by a putative (mitochondrial or endomembrane) L-gulono-1,4-lactone dehydrogenase. Methyl jasmonate-inducible genes of *Arabidopsis* (At4g26850 and At5g56490) (Zimmermann et al., 2004) and homologs of methyl jasmonate-inducible genes in tobacco cells (At5g28840, At3g09440, At2g46750 and At5g50400; Wolucka et al., 2005; and Wolucka, 2005, respectively) are underlined. Enzymes that are able to process the intermediates of both pathways are indicated in the middle.

At2g46760, At1g32300, At5g56490, At5g11540) have been identified in the *Arabidopsis* genome (Wolucka and Van Montagu, 2003). Altogether, these experimental facts point to a more complex picture of the *de novo* biosynthesis of vitamin C in plants, and led to the proposal of the L-gulose pathway (Fig. 2).

3. The enzymatic functions of the plant VTC2 protein

The vitamin C-deficient *vtc2* mutants of *Arabidopsis thaliana* were generated by random ethyl methanesulfonate mutagenesis and isolated in an ozone sensitivity-based screen (Conklin et al., 2000). The *vtc2* Arabidopsis mutants contain about 25% of the wild-type vitamin C levels, and have a point mutation in the At4g26850 (*VTC2*) gene (GenBank accession number AF508793) that was identified by positional cloning (Jander et al., 2002). Another gene encoding a paralog of the VTC2 protein (At5g55120; 76% identity) exists in the *A. thaliana* genome, and VTC2 orthologs are present in other plants. It is worthy to note that the closest VTC2 homolog of *Oryza sativa* shows only 68% identity with the *Arabidopsis* protein (Table 1). VTC2-like proteins are found in Chlorophyceae (*Chlamydomonas reinhardtii*) but apparently not in other groups of algae as, for example, diatoms (*Thalassiosira pseudonana*), red algae (*Cyanidioschyzon merolae*), and certain Prasinophyta (*Ostreococcus tauri*). The only bacterial homolog of the *Arabidopsis* VTC2 protein (26% identity) is present in a chemolithoautotrophic β-proteobacterium *Thiobacillus denitrificans* but its function is unknown. Other bacteria, protists, fungi and some insects are devoid of VTC2 homologs. Interestingly, VTC2-like proteins can be found in vitamin C-synthesizing vertebrates, but also in species that are unable of vitamin C synthesis because of the lack of the last enzyme L-gulonolactone oxidase, such as humans and other primates (30% identity), the *Danio rerio* fish, certain insects

Table 1
The proposed plant proteins for the *de novo* synthesis of L-ascorbic acid and the closest homologs in bacteria, algae and other eukaryotes

Arabidopsis proteins for the *de novo* biosynthesis of L-ascorbic acid[a] (% identity with the closest ortholog of *Oryza sativa*)	The closest homologs in algae[b] (% identity with the *Arabidopsis* protein)				The closest homologs in other organisms[c] (% identity with the *Arabidopsis* protein)	
	Chlorophyceae *Chlamydomonas reinhardtii.*(%)	Prasinophyta *Ostreococcus tauri.*(%)	Rhodophyta *Cyanidioschyzon merolae* (%)	Heterokontophyta *Thalassiosira pseudonana.*(%)	Other eukaryotes (%)	Bacteria.(%)
Phosphomannose isomerase, At1g67070 (53)	44	41	37	40	*Bos taurus* (42)	*Streptomyces coelicolor* (32)
Phosphomannomutase, *At2g45790* (81)	56	69	55	62	*Schizosaccharomyces pombe* (62)	*Sphingomonas sp.*(26)
GDP-mannose Pyrophosphorylase (VTC1), At2g39770 (87)	65	–	60	–	*Canis familiaris* (61)	*Geobacillus thermodenitrificans* (36)
GDP-D-mannose 3″,5″-epimerase, At5g28840 (91)	74	70	65	61	*Trichomonas vaginalis* (62)	*Acidobacteria* bacterium (51)
GDP-L-galactose : hexose 1-phosphate guanylyltransferase (VTC2), At4g26850 (68)	48	–[d]	–	–	*Danio rerio* (29)	*Thiobacillus denitrificans* (27)
Putative phosphodiesterase/metallophosphatase, At5g50400[e] (65)	30	32	28	33	*Dictyostelium discoideum* (34)	*Myxococcus xanthus* (27)
L-galactose 1-P/*myo*-inositol 1-P monophosphatase (VTC4), At3g02870 (70)	52	48	38	41	*Drosophila melanogaster* (44)	*Pelobacter carbinolicus* (39)
L-galactose dehydrogenase, At4g33670 (74)	28[f]	24	46	39	*Strongylocentrotus purpuratus* (50)	*Pseudoalteromonas atlantica* (47)
L-galactono-1,4-lactone dehydrogenase, At3g47930 (70)	–	48	46	39	*Sus scrofa* (26)	*Brevibacterium linens* (partial 228 aa) (30)
L-gulono-1,4-lactone dehydrogenase, At2g46740 (52)	22	–	–	–	*Aspergillus terreus* (25)	*Hahella chejuensis* (27)

[a] BLAST searches of the *Arabidopsis* genome were performed at the site: http://www.arabidopsis.org/cgi-bin/Blast.

[b] BLAST searches of algal genomes were performed at the following sites: http://genome.jgi-psf.org/Ostta4/Ostta4.home.html (*Ostreococcus tauri*); http://genome.jgi-psf.org/Chlre3/Chlre3.home.html (*Chlamydomonas reinhardtii);* http://merolae.biol.s.u-tokyo.ac.jp/blast/blast.html (*Cyanidioschyzon merolae*); http://genome.jgi-psf.org/cgi-bin/runAlignment?db=thaps1&advanced=1 (*Thalassiosira pseudonana*).

[c] BLAST searches were performed at the site: http://www.ncbi.nlm.nih.gov/BLAST/.

[d] A VTC2 homolog is present in a related species *Ostreococcus lucimarinus* (54% identity).

[e] The At5g50400 putative phosphodiesterase/metallophosphatase (Wolucka, 2005) is homologous to the late methyl jasmonate-inducible gene of *Nicotiana tabacum*, whose transcription was strongly enhanced in methyl jasmonate-treated tobacco BY2 cells, at the same time as the GDP-mannose 3″,5″-epimerase (At5g28840) and a putative L-gulono-1,4-lactone dehydrogenase (At2g46750) (Wolucka et al., 2005).

[f] The *Chlamydomonas reinhardtii* protein is related to the At1g04690 potassium channel β-subunit of *Arabidopsis thaliana* (56% identity).

(*Drosophila sp.*), and *Caenorhabditis* nematodes (24% identity).

The VTC2 gene function remained elusive until very recently. Two apparently contradictory papers describe the VTC2 enzyme activity either as a GDP-L-galactose/GDP-D-glucose phosphorylase (Linster et al., 2007) or a GDP-L-galactose : hexose 1-phosphate guanylyltransferase (EC 2.7.7.12) (Laing et al., 2007). Both groups based their research on bioinformatics data indicating that the VTC2 protein belongs to the branch III of the HIT (histidine triad) superfamily of nucleotide hydrolases and transferases. The VTC2 protein contains a HxHxQxx motif (the HLHFQAY sequence) that is characteristic for the branch III specific nucleoside monophosphate transferase/galactose 1-phosphate uridylyltransferase (GalT) enzymes (Brenner, 2002). The reaction of branch III nucleotide transferases involves the formation of a covalent nucleotidylated enzyme intermediate with the second His residue of the HxHxQxx motif. The enzyme-bound nucleoside monophosphate is then transferred to a final acceptor such as inorganic phosphate (phosphorylases) or phosphorylated second substrate (transferases).

The recombinant VTC2 enzyme of *Arabidopsis thaliana* and *Actinidia chinensis* has both phosphorylase and transferase activities *in vitro* (Laing et al., 2007). The phosphorylase activity was showed to catalyze the following reversible reactions (Linster et al., 2007) (reactions 1 and 2):

$$\text{GDP-L-galactose} + P_i \leftrightarrow \text{L-galactose 1-phosphate} + \text{GDP} \quad (1)$$

$$\text{GDP-D-glucose} + P_i \leftrightarrow \text{D-glucose 1-phosphate} + \text{GDP} \quad (2)$$

The GDP-L-galactose substrate was generated by using the recombinant GDP-D-mannose 3″,5″-epimerase (Wolucka et al., 2001; Major et al., 2005). The phosphorylase assay included an anion-exchange HPLC separation and spectrophotometric measurement of the GDP product. GDP-D-mannose was a poor substrate, whereas UDP-glucose, UDP-galactose and ADP-glucose were not utilized by the VTC2 enzyme. GDP-L-Fucose was not tested. Based on the reverse-phase HPLC profile, GDP-L-gulose that is formed in the epimerase reaction (Wolucka and Van Montagu, 2003; Major et al., 2005), apparently did not undergo significant phosphorolysis in the presence of the VTC2 protein, although the position of the GDP product in the reverse-phase HPLC profile was not indicated.

The VTC2 phosphorylase has a low K_m value (10 μM and 4.4 μM), a high turnover rate k_{cat} (64 s^{-1} and 23 s^{-1}), and a similar specificity constant k_{cat}/K_m (6.3×10^6 s^{-1} M^{-1} and 5.7×10^6 s^{-1} M^{-1}) with the GDP-L-galactose and GDP-D-glucose substrates, respectively, in the presence of 5 mM inorganic phosphate (Linster et al., 2007).

On the other hand, an indirect enzyme assay developed by Laing et al. (2007) allowed to measure the guanylyltransferase activity of the VTC2 enzyme (EC 2.7.7.12), according to the following reaction (reaction 3):

$$\text{GDP-L-galactose} + \text{D-mannose 1-phosphate} \rightarrow \text{GDP-D-mannose} + \text{L-galactose 1-phosphate} \quad (3)$$

(It is not clear if the reaction 3 is irreversible, as claimed by the authors.)

In this test, the release of L-galactose 1-phosphate from the GDP-L-galactose substrate was determined by using two coupled enzymatic reactions catalyzed by the L-galactose 1-phosphate/*myo*-inositol 1-phosphate phosphatase and the NAD-dependent L-galactose dehydrogenase, respectively, and a spectrophotometric measurement of the reduced NAD.

The K_m values of the VTC2 guanylyltransferase measured for D-mannose 1-phosphate, inorganic phosphate and pyrophosphate were 0.11, 4.4 and 0.16 mM, respectively, whereas the corresponding V_{max} values were 0.17 nmol s^{-1} (μg of protein)$^{-1}$, 0.12 nmol s^{-1} (μg of protein)$^{-1}$, and 0.032 nmol s^{-1} (μg of protein)$^{-1}$, respectively.

The indirect enzymatic assay for the VTC2 activity is a subject of error, in particular, at low substrate concentrations (see Fig. 3 in: Laing et al., 2007). Moreover, there is some inconsistency in the reported V_{max} and the reaction velocity values for D-mannose 1-phosphate (0.17 nmol s^{-1} (μg of protein)$^{-1}$ and 0.33 nmol s^{-1} (μg of protein)$^{-1}$, respectively). The K_m value for GDP-L-galactose was not determined but, on the basis of the presented data, it can be roughly estimated as 10 μM (Laing et al., 2007).

In spite of these shortcomings, the kinetic data reported by Laing et al. (2007) are similar to those reported by Linster et al. (2007) for the forward VTC2 phosphorylase reaction. The approximate turnover rate (k_{cat}) of the VTC2 enzyme with the inorganic phosphate and GDP-L-galactose substrates was 6 s^{-1} (Laing et al., 2007) vs. 64 s^{-1} (Linster et al., 2007). In the guanylyltransferase reaction, the k_{cat} value of the VTC2 protein for D-mannose 1-phosphate was about 9 s^{-1}. Accordingly, the specificity constant k_{cat}/K_m for the D-mannose 1-phosphate was at least 60 times higher compared to inorganic phosphate (8.1×10^4 s^{-1} M^{-1} and 1.4×10^3 s^{-1} M^{-1}, respectively), indicating that D-mannose 1-phosphate is a better substrate for the VTC2 enzyme.

Importantly, the VTC2 transferase has broad substrate specificity and can use D-glucose 1-phosphate (reaction 4) and other hexose phosphates, as guanylyl acceptors.

$$\text{GDP-L-galactose} + \text{D-glucose 1-phosphate} \rightarrow \text{GDP-D-glucose} + \text{L-galactose 1-phosphate} \quad (4)$$

The guanylyltransferase activity of the VTC2 enzyme preserves the energy of the phosphodiester linkage of GDP-sugar derivatives and, importantly, does not release GDP which is a potent inhibitor of the GDP-D-mannose 3″,5″-epimerase (Wolucka and Van Montagu, 2003). The enzyme uses D-glucose 1-phosphate as a substrate that,

unlike D-mannose 1-phosphate, is abundant in plant cells. In summary, the above-discussed kinetic data and physiological considerations strongly suggest that the VTC2 protein acts as a unique GDP-L-galactose/GDP-D-glucose: hexose 1-phosphate guanylyltransferase of broad specificity. It is possible that the main reaction catalyzed by the VTC2 enzyme in photosynthetically active plant cells is the reaction 4 (see above). What could be the fate of GDP-D-glucose produced in the VTC2-catalyzed reaction is discussed in the next section.

Interestingly, an *Agrobacterium*-mediated transient overexpression of the *Actinidia* VTC2 protein in tobacco leaves resulted in 50-folds higher guanylyltransferase activity and a modest (2.5–3-fold) increase of L-ascorbic acid levels (Laing et al., 2007). A similar limited increase of ascorbic acid content was reported for transgenic *Arabidopsis* plants overexpressing the D-galacturonic acid reductase of *Fragaria sp*. that converts D-galacturonic acid to L-galactono-1,4-lactone in a salvage pathway for L-ascorbic acid (Agius et al., 2003). These observations point to the existence of ascorbic acid homeostasis in plants. It is believed that ascorbic acid biosynthesis is tightly controlled via feedback inhibition (Imai et al., 1999) at the GDP-mannose 3″,5″-epimerase level (Wolucka and Van Montagu, 2003), and, possibly, by other mechanisms, still to be determined.

Little is known about specific inhibitors of the VTC2 enzyme, the effects of *vtc2* mutations on the enzyme activity, the proteins that interact with the VTC2 protein and the cellular localization of the latter, although such knowledge would help unravel possible metabolic channeling and physiological functions of the VTC2 enzyme.

In conclusion, the VTC2 GDP-L-galactose/GDP-D-glucose : hexose 1-phosphate guanylyltransferase is a relatively unspecific enzyme. The VTC2 enzyme uses at least two different GDP-hexoses with a *trans* configuration of 2″- and 3″- hydroxyl groups of the hexosyl residue as guanylyl donors, and different hexose phosphates, inorganic phosphate and pyrophosphate as guanylyl acceptors, with a net preference for hexose 1-phosphates. Thus, the VTC2 enzyme catalyzes both guanylyltransferase and phosphorylase reactions, and at least the latter reaction is reversible. Consequently, the first specific and committed step of the L-galactose pathway is the irreversible hydrolysis of L-galactose 1-phosphate catalyzed by the At3g02870 L-galactose 1-phosphate/*myo*-inositol 1-phosphate phosphatase (VTC4) (Laing et al., 2004; Conklin et al., 2006) and, possibly, other enzymes.

Due to its guanylyltransferase activity, the VTC2 enzyme participates in the formation of GDP-D-mannose and other GDP-hexoses for the synthesis of polysaccharides, including glucomannans (Liepman et al., 2005), and/or glycoproteins. In the presence of D-mannose 1-phosphate, both the VTC2 (guanylyltransferase) and the VTC1 (GDP-mannose pyrophosphorylase) enzymes produce GDP-D-mannose. It is possible that GDP-D-mannose formed by the VTC2 enzyme is utilized for the synthesis of distinct polymers than the GDP-mannose produced by the VTC1 pyrophosphorylase. In agreement, the *vtc1* and *vtc2* mutants of *Arabidopsis thaliana* have different phenotypes (Olmos et al., 2006), although similar low ascorbic acid contents (25–30% of the wild-type levels) (Conklin et al., 1996 and Conklin et al., 2000). Consequently, the observed phenotypic differences between the *vtc* mutants can hardly be a direct consequence of lower ascorbic acid levels, and are, probably, related to specific changes in the cell-wall/glycoprotein profiles of the mutants.

Laing et al. (2007) speculated about a possible role of cell-wall mannans as a source of D-mannose for the synthesis of L-ascorbic acid. A D-mannose salvage pathway could provide D-mannose 1-phosphate for the GDP-L-galactose-dependent reaction of the VTC2 guanylyltransferase. The so formed GDP-D-mannose is then converted by the GDP-D-mannose 3″,5″-epimerase to GDP-L-galactose, and the latter compound can enter another cycle.

4. The extended VTC2 cycle and its possible role in plant physiology

Although very convincing, the VTC2 cycle proposed by Laing et al. (2007) does not solve the problem of the carbon source for the L-galactose pathway to vitamin C, and, in general, of the origin of D-mannose in plant cells in the absence of phosphomannose isomerase activity. We propose that the missing enzyme could be the long known but still unidentified GDP-D-mannose 2″-epimerase which reversibly converts GDP-D-glucose to GDP-D-mannose (Feingold, 1982; Elbein, 1969). The extended VTC2 cycle would then comprise: a GDP-D-mannose 2″-epimerase, the GDP-D-mannose 3″,5″-epimerase, and the GDP-L-galactose/GDP-D-glucose guanylyltransferase (VTC2) (Fig. 3). GDP-D-glucose that can be formed from glucose 1-phosphate in a VTC2-catalyzed reaction (reaction 4), strongly inhibits the GDP-D-mannose 3″,5″-epimerase (Wolucka and Van Montagu, 2003). Therefore, the 2″-epimerization of GDP-D-glucose could not only produce GDP-mannose for the synthesis of polymers and of vitamin C, but, simultaneously, activate the GDP-D-mannose 3″,5″-epimerase.

Labeling experiments with exogenous [^{14}C] hexoses showed that D-mannose is a more efficient precursor for L-ascorbic acid synthesis than D-glucose in plants (Wheeler et al., 1998). This could be due, however, to the existence of a robust salvage pathway for D-mannose involving a hexokinase (or a 1-kinase) and a phosphomannose mutase that furnish D-mannose 1-phosphate to the VTC2 cycle. Indeed, D-mannose is toxic for plants (Herold and Lewis, 1977), inhibits fruit ripening (Watkins and Frenkel, 1987), and triggers the cell death program (Stein and Hansen, 1999). Therefore, the excess D-mannose must be rapidly detoxified. However, D-mannose 6-phosphate isomerase activity, which converts D-mannose 6-phosphate to fructose 6-phosphate in a reversible reaction, and was proposed to be the

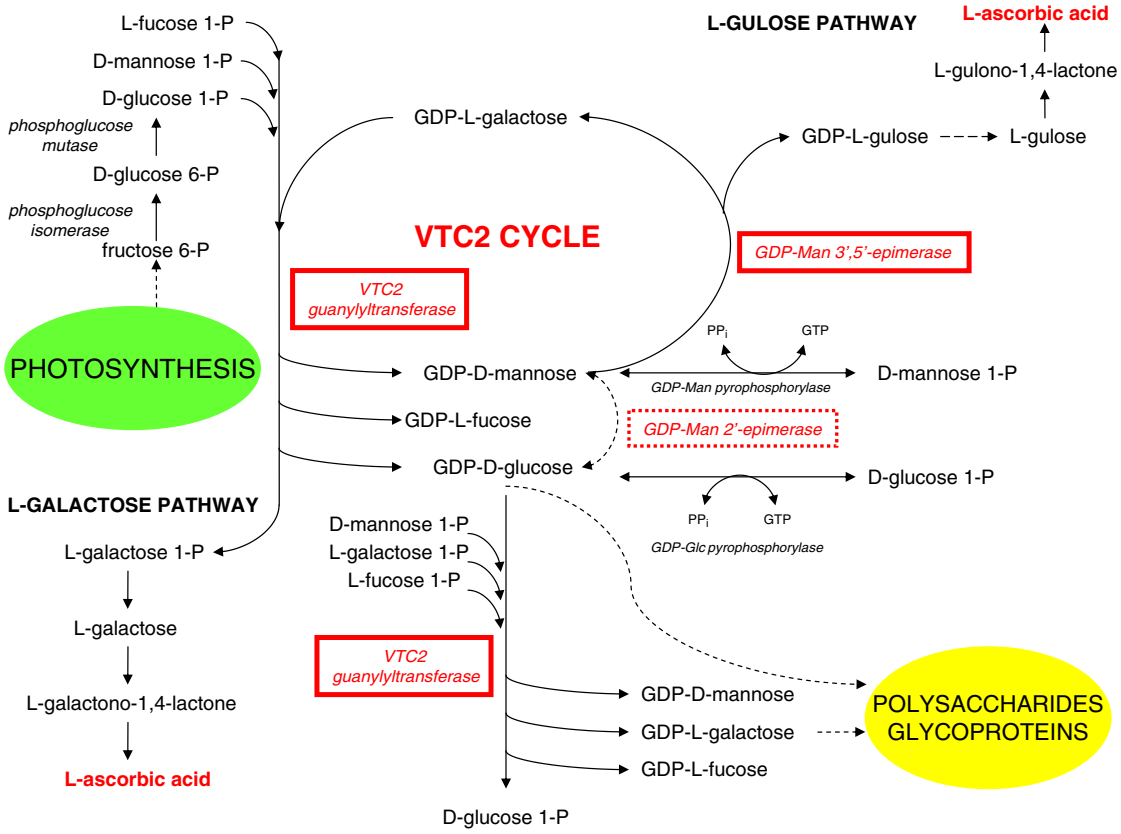

Fig. 3. The proposed extended VTC2 cycle and its central role in the *de novo* biosynthesis, salvage and detoxification pathways of plants. The GDP-L-galactose/GDP-D-glucose : hexose 1-phosphate guanylyltransferase (VTC2) (Jander et al., 2002; Laing et al., 2007)/GDP-D-mannose 3″,5″-epimerase cycle proposed originally by Laing et al. (2007) could be further extended, taking into account the existence in plants of a GDP-D-mannose 2″-epimerase activity, and the fact that the VTC2 enzyme uses both GDP-L-galactose and GDP-D-glucose (Linster et al., 2007) as guanylyl donors. The extended VTC2 cycle has biosynthetic, regulatory and detoxification functions, and couples the synthesis of vitamin C and the formation of GDP-sugars for plant polymers with the photosynthetic carbon flow and salvage pathways for the utilization of hexoses. Hexose 1-phosphates derived from either the general metabolism and, in particular, photosynthesis (D-glucose 1-P), or the degradation of polysaccharides/glycoproteins (D-glucose 1-P, D-mannose 1-P, L-fucose 1-P, L-galactose 1-P) are converted to the corresponding GDP-hexoses by the VTC2 guanylyltransferase that uses either GDP-L-galactose or GDP-D-glucose as a guanylyl donor, with a concomitant release of L-galactose 1-phosphate and D-glucose 1-phosphate, respectively. L-Galactose 1-phosphate enters the L-galactose pathway for the synthesis of L-ascorbic acid (Wheeler et al., 1998), whereas D-glucose 1-phosphate can undergo another cycle. The newly formed GDP-hexoses can be used for the synthesis of polymers, or be recycled after a prior 3″,5″-epimerization of GDP-D-mannose to GDP-L-galactose catalyzed by the GDP-D-mannose 3″,5″-epimerase (Wolucka et al., 2001), and a 2″-epimerization of GDP-D-glucose by a still unidentified plant GDP-D-mannose 2″-epimerase, followed by a 3″,5″-epimerization of the GDP-D-mannose product. The GDP-D-mannose 3″,5″-epimerase forms also GDP-L-gulose which, apparently, is not a substrate for the VTC2 guanylyltransferase, and could be used for the synthesis of vitamin C *via* the L-gulose pathway (Wolucka and Van Montagu, 2003). The priming reactions to start the VTC2 cycle could involve a GDP-D-glucose pyrophosphorylase and a GDP-D-mannose 2″-epimerase to convert D-glucose 1-phosphate to GDP-D-glucose and, finally, to GDP-D-mannose; or, as proposed by Laing et al. (2007), a GDP-D-mannose pyrophosphorylase, probably the VTC1 protein (Conklin et al., 1999). The VTC2 guanylyltransferase discriminates between GDP-hexoses with *trans*- or *cis*-configuration of the 2″- and 3″-hydroxyl groups of the hexosyl residue and, apparently, does not use the *cis*-compounds such as GDP-D-mannose and GDP-L-gulose (Linster et al., 2007). L-Fucose derivatives have a *trans*-configuration of the above-mentioned groups and could, probably, enter the VTC2 cycle, as shown in the scheme, although this is speculative.

first enzyme in the pathway for vitamin C (Wheeler et al., 1998), is not detectable in many plants (Gao et al., 2005; Zhu et al., 2005), except in mannitol-producing species, such as celery (Rumpho et al., 1983; Everard et al., 1997). The apparent lack of phosphomannose isomerase activity combined with a strong hexokinase activity lead to the accumulation of D-mannose 6-phosphate and the depletion of ATP and P_i reserves in the presence of exogenously supplied D-mannose. These changes are thought to be responsible for D-mannose toxicity in plants, although other mechanisms, including sugar-sensing, cannot be excluded.

The *Arabidopsis* genome contains one copy of a phosphomannose isomerase gene (At1g67070). The phosphomannose isomerase expression is very weak and induced in senescence and dark (Fujiki et al., 2001). Like some other vitamin C-related genes (Wolucka et al., 2005), the isomerase is stimulated by methyl jasmonate (Zimmermann et al., 2004). Considering a high demand for D-mannose derivatives for the synthesis of structural components such as non-cellulosic polysaccharides, glycoproteins, glycolipids, but also L-ascorbic acid, the reported lack of phosphomannose isomerase activity in the majority of plant tissues is puzzling. Another putative entry for D-mannose

derivatives is an unknown GDP-D-mannose 2″-epimerase activity suggested by Feingold (1982), based on the results of *in vitro* labeling experiments of Elbein (1969). This enigmatic enzyme would be responsible for a conversion of GDP-D-mannose into GDP-D-glucose, thus linking the D-glucose and D-mannose metabolism at the level of sugar nucleotides. Although direct evidence for the existence of GDP-D-mannose 2″-epimerase is still lacking, such enzyme activity could help resolve the problem of carbon source for the synthesis of D-mannose-derived compounds in plants.

In contrast to D-mannose which is confined to D-mannose pathways due to the lack of phosphomannose isomerase activity, exogenously supplied [^{14}C] D-glucose is diverted to many pathways, and only a fraction is devoted to vitamin C synthesis. This fact probably explains the observed lower efficiency of labeling with [^{14}C] D-glucose compared to [^{14}C] D-mannose (1% and 10% of the total radioactivity in L-ascorbic acid, respectively) (Wheeler et al., 1998), but does not exclude D-glucose derivatives as precursors for ascorbic acid synthesis. It is possible, therefore, that the main carbon flow to vitamin C in plants under normal conditions does not involve D-mannose phosphates and D-mannose 6-phosphate isomerase as proposed by Wheeler et al. (1998), but D-glucose phosphates that are converted to L-hexoses for L-ascorbic acid synthesis *via* the VTC2 cycle and a GDP-D-mannose 2″-epimerase (see the Legend to Fig. 3).

In this way, the products of photosynthesis (fructose 6-phosphate and glucose phosphates) can directly enter the VTC2 cycle with no need for D-mannose 6-phosphate isomerase activity: fructose 6-phosphate is isomerized by a D-glucose 6-phosphate isomerase to D-glucose 6-phosphate, and the latter is converted by a phosphoglucose mutase to D-glucose 1-phosphate which via the VTC2 cycle provides a carbon skeleton for L-galactose 1-phosphate and, finally, L-ascorbic acid. In such scenario, the VTC2 guanylyltransferase in cooperation with the GDP-mannose 3″,5″-epimerase and a putative GDP-D-mannose 2″-epimerase, could serve as a formidable engine for the production of the L-galactose 1-phosphate precursor of vitamin C, but also of GDP-hexoses (GDP-D-mannose, GDP-D-glucose, GDP-L-galactose, GDP-L-gulose, and, perhaps, GDP-L-fucose) for glucomannan and other polymer synthesis, while conserving the energy of the phosphodiester linkage.

In summary, the extended VTC2 cycle and the L-galactose pathway form, probably, the major *de novo* route for L-ascorbic acid synthesis that uses photosynthetic intermediates, fructose and glucose phosphates. On the other hand, the L-gulose shunt could be used in conditions of high stress in order to escape the regulatory constraints of the VTC2 cycle (Fig. 2). Apart from the GDP-D-mannose 3″,5″-epimerase, the L-gulose pathway may involve a GDP-D-glucose or a GDP-D-mannose pyrophosphorylases, the putative phosphodiesterase/metallophosphatase (At5g50400) (Wolucka, 2005), L-galactose dehydrogenase or another protein similar to the At1g04690 potassium channel β-subunit aldoreductase (Table 1; note 6), L-gulono-1,4-lactone dehydrogenases (At2g46740, At2g46750, At2g46760, At1g32300, At5g56490, At5g11540) (Wolucka and Van Montagu, 2003), and, perhaps, other, still unknown proteins (Fig. 2). The role of these proteins in the L-gulose pathway remains to be elucidated.

5. Evolution of vitamin C biosynthesis in plants

The enzymes for the *de novo* synthesis of L-ascorbic acid are well conserved in both dicot and monocot plants, with the most conserved protein being the GDP-mannose 3″,5″-epimerase (the *Arabidopsis* epimerase shares 91% identity with the *Oryza sativa* ortholog) (Table 1). The enzymes for the general D-mannose metabolism, such as phosphomannose isomerase, phosphomannose mutase and GDP-mannose pyrophosphorylase have close orthologs in mammals and other eukaryotes (about 60% identity), thus pointing to their ancient history and common functions in plants and animals (Table 1). On the contrary, the proteins for the dedicated steps of the plant *de novo* pathways for vitamin C, such as the GDP-D-mannose 3″,5″-epimerase and the downstream enzymes, have no close homologs in higher animals, with the exception of L-galactose 1-phosphate/*myo*-inositol 1-phosphate phosphatase and L-galactose dehydrogenase, suggesting that the latter proteins could possibly be involved in other processes. Indeed, manipulation of L-galactose dehydrogenase expression had barely an effect on plant vitamin C levels (Gatzek et al., 2002).

Recently, the genome sequences of four algae have been released: two green algae *Chlamydomonas reinhardtii* (Chlorophyta, Chlorophyceae) (http://www.jgi.doe.gov) and *Ostreococcus tauri* (Chlorophyta, Prasinophyceae) (Derelle et al., 2006), a red alga *Cyanidioschyzon merolae* (Rhodophyta) (Matsuzaki et al., 2004), and the photosynthetic diatom *Thalassiosira pseudonana* (Heterokontophyta) (Armbrust et al., 2004). No systematic study has been conducted until now on the presence of ascorbic acid and related compounds in different groups of algae. Radiolabelling experiments showed that members of green photosynthetic Chlorophyceae algae (*Chlorella pyrenoidosa*) and of a closely related non-photosynthetic Trebouxiophyceae group (*Prototheca sp.*) convert D-glucose into ascorbic acid without inversion of the carbon chain. In contrast, an inversion pathway operates in other algal classes such as golden algae (*Ochromonas danica*) and diatoms (*Cyclotella cryptica*) of the Heterokontophyta group, and in Euglenophyta protists (*Euglena gracilis*) (reviewed in: Loewus, 1999). These observations suggest the existence of at least two different pathways for L-ascorbic acid in algae.

Inspection of the algal genomes by using the vitamin C-related protein sequences of *Arabidopsis thaliana* has revealed some surprising facts (Table 1). Firstly, all algae

contain a close homolog of the plant GDP-mannose 3″,5″-epimerase (61–74% identity), indicating that the epimerase sequence was acquired by a proto-alga or earlier in the evolution. Interestingly, modern cyanobacteria do not have GDP-mannose 3″,5″-epimerase-like sequences. It seems unlikely, therefore, that the epimerase gene could be acquired from a cyanobacterium-like endosymbiont. Remarkably, sequences similar to the *Arabidopsis* GDP-mannose 3″,5″-epimerase are present in other groups of bacteria, with the closest homolog present in an acidobacterium (51% identity) (Table 1), but also in a eukaryotic flagellated protozoan *Trichomonas vaginalis* (62% identity).

Secondly, the algal genomes of Prasinophyta, Rhodophyta and Heterokontophyta groups, except Chlorophyceae (*Chlamydomonas reinhardtii*), contain genes that are closely related to the plat L-galactono-1,4-lactone dehydrogenase (39–48% of identity) (Table 1). In contrast, the green photosynthetic microalga *Chlamydomonas reinhardtii*, but not the three other algae, harbors at least one protein that is homologous (22% identity) to the putative L-gulono-1,4-lactone dehydrogenase At2g46740 of the L-gulose pathway for vitamin C in plants (Wolucka and Van Montagu, 2003). This observation provides indirect evidence for the involvement of the putative L-gluono-1,4-lactone dehydrogenases in the *de novo* synthesis of vitamin C. Clearly, L-galactonolactone dehydrogenase gene was retained and well-conserved in higher plants, but highly mutated or lost in green algae, probably after the acquisition of the L-gluono-1,4-lactone dehydrogenase gene. BLAST searches of eukaryotic and prokaryotic genomes revealed that only few species of bacteria contain a protein similar to the plant L-gulono-1,4-lactone dehydrogenases, and the closest homolog is present in a marine proteobacterium *Hahella chejuensis* (27% identity) that is closely associated with algal populations, and in some actinobacteria (Wolucka and Communi, 2006). It seems probable, therefore, that an ancient L-gulono-1,4-lactone dehydrogenase gene of a common ancestor of green algae and land plants could be acquired *via* horizontal gene transfer from an ancient marine eubacterium.

Chlamydomonas reinhardtii is closely related to *Chlorella* and *Volvox* algae, that all belong to the same Chlorophyceae group of Viridiplantae. As mentioned earlier, ascorbic acid is synthesized in *Chlorella sp. via* a non-inversion pathway that is thought to involve GDP-mannose 3″,5″-epimerase (Running et al., 2003). Indeed, the GDP-mannose 3″,5″-epimerase protein of *C. reinhardtii* is very similar to the *Arabidopsis* enzyme (74% identity) (Table 1). Significantly, gulose residues that are formed by the action of GDP-mannose 3″,5″-epimerase (Wolucka and Van Montagu, 2003), were detected in the cell-wall of *Volvox* (Mengele and Sumper, 1992). In addition to the L-gulono-1,4-lactone dehydrogenase and GDP-Man 3″,5″-epimerase proteins, the *C. reinhardtii* alga possesses also a GDP-mannose pyrophosphorylase and a VTC2-like sequence (65% and 48% identity with the *Arabidopsis* proteins, respectively). In contrast, L-gulono-1,4-lactone dehydrogenase, VTC2 and GDP-mannose pyrophosphorylase proteins are lacking in the sequenced Prasinophyta, Rhodophyta and Heterokontophyta algae, with the exception of two GDP-mannose pyrophosphorylase orthologs of the red alga *Cyanidioschyzon merolae* (Table 1). These facts suggest that, like in plants, the L-gulono-1,4-lactone dehydrogenase protein of Chlorophyceae algae (*C. reinhardtii*) is the only enzyme responsible for the last step of vitamin C synthesis *via* a non-inversion *de novo* pathway (Wolucka and Van Montagu, 2003).

On the other hand, the L-galactono-1,4-lactone dehydrogenase proteins of Prasinophyta, Rhodophyta and Heterokontophyta algae could be responsible for the synthesis of L-ascorbic acid via an inversion pathway that probably involves UDP-D-glucose dehydrogenase and glucuronic acid/galacturonic acid intermediates.

In conclusion, plant genes involved in the dedicated steps of the *de novo* pathways for vitamin C, such as GDP-mannose 3″,5″-epimerase, VTC2-guanylyltransferase and L-gulono-1,4-lactone dehydrogenase, are, probably, of eubacterial origins, and the two latter sequences could be acquired by a common ancestor of plants (green algae and land plants) later in the evolution.

Endosymbiosis and horizontal gene transfer are major driving forces in the evolution and diversification of plants and algae. A primary endosymbiotic event, the engulfment of a cyanobacterium-like organism by a proto-alga and the establishment of permanent endosymbiosis, led to the development of modern plastids. Whereas a cannibalism-like secondary endosymbiosis, *i.e.* the engulfment of an alga, could occur in other groups of algae, such event apparently did not take place in an ancestor of green algae and plants. However, it is possible that enhanced permissiveness to harbor eubacteria persisted in a plant ancestor and led to the development of gene transfer mechanisms, the remnants of which can still be observed in the case of *Agrobacterium*-plant host interactions. The multiplicity and the complexity of bacterial and mycorrhizal symbioses of modern algae and plants point out the ancient history of such interactions. The capacity of a plant ancestor to assemble genes of eubacterial origin probably played a major role in the evolution of extraordinarily rich panoply of biochemical pathways that are characteristic of modern plants. Perhaps, such gene transfer permissiveness was necessary for a plant ancestor to capture genetic information while leading a sessile lifestyle.

It is tempting to speculate that a common ancestor of algae and plants had acquired an eubacterial gene for GDP-mannose 3″,5″epimerase via endosymbiosis-mediated horizontal gene transfer. Subsequent acquisition of ancient L-gulono-1,4-lactone dehydrogenase and VTC2 guanylyltransferase genes from ancient eubacteria could be a decisive step in the evolution of *de novo* biosynthesis pathways for vitamin C in the plant lineage. Once

acquired, the L-gulono-1,4-lactone dehydrogenase and the VTC2 genes could undergo further evolution via gene duplication and mutations in the ancestor of modern higher plants. Indeed, two VTC2 paralogs (At4g26850 and At5g55120) and six closely related L-gulono-1,4-lactone dehydrogenase-like proteins (At2g46740, At2g46750, At2g46760, At1g32300, At5g56490, At5g11540) are present in *A. thaliana*. Three of the L-gulono-1,4-lactone dehydrogenase genes (At2g46740, At2g46750, At2g46760) are paralogs (85% of identity) located in tandem on chromosome 2. The corresponding gene products are predicted to differ in their cellular location (mitochondrial *versus* endomembrane system) probably due to the acquisition of differential targeting sequences during the evolution of higher plants (Fig. 2). The development of very efficient L-galactose/L-gulose routes for L-ascorbic acid has led to increased levels of L-ascorbic acid for panoply of emerging, evolutionary new vitamin C-dependent enzymes and processes in the history of higher plants. These changes have probably played an important role in the separation of the *Viridiplantae* lineage and the evolution of higher plants.

The role of symbiosis in the acquisition of vitamins and, perhaps, in the development of capacities to synthesize them, can still be observed in modern algae in the case of cobalamine cofactor (vitamin B_{12}) (Croft et al., 2005). Vitamin B_{12} is acquired by certain algae from bacterial symbionts because of a non-functional biosynthetic pathway for cobalamine with a concomitant presence of algal enzymes that require the vitamin as a cofactor. Other algae do not show vitamin B_{12} auxotrophy, either because they are able to synthesize the cofactor, or because, like plants, they do not have vitamin B_{12}-dependent enzymes.

The recent discovery of a functional bacterial gene for vitamin C synthesis, the L-gulono-1,4-lactone dehydrogenase of the *Mycobacterium tuberculosis* pathogen, and the fact that similar proteins are present in many bacterial pathogens and symbionts (Wolucka and Communi, 2006), further support the hypothesis of a eubacterial origin of vitamin C–related genes in the evolution of higher eukaryotes. A common ancestor of green algae and plants had assembled evolutionary novel VTC2 and L-gulono-1,4-lactone dehydrogenase genes, in addition to the more ancient algal GDP-mannose 3″,5″-epimerase, thus forming a formidable enzymatic machinery for an extremely efficient production of L-ascorbic acid. The enzymatic arsenal for L-ascorbic acid has been further multiplied in the higher plant lineage *via* duplication of genes for L-gulono-1,4-lactone dehydrogenase and VTC2-guanylytransferase (and perhaps others), and it is possible that these events determined the evolutionary success of higher plants.

Further studies are necessary to identify algal pathways for vitamin C and to characterize the enzymes involved. This knowledge would help better understand evolutionary origins, pathways and functions of L-ascorbic acid in plants.

6. Further perspectives

The VTC2 guanylyltransferase and the GDP-mannose 3″,5″-epimerase form clearly the heart of the *de novo* synthesis of vitamin C in plants. Little is known about the regulation of plant enzymes for vitamin C synthesis. The GDP-mannose 3″,5″-epimerase activity was shown to undergo a fine control (Wolucka and Van Montagu, 2003). The *Arabidopsis* 3″,5″-epimerase co-purified with a Hsc70.3 heat-shock cognate protein, thus suggesting the existence of protein-protein interactions that may affect the enzyme activity. The partially purified, native epimerase of *Arabidopsis*, but not the recombinant enzyme, was slightly inhibited by L-ascorbic acid, indicating that the enzyme could be the target of feedback inhibition, perhaps, with the participation of some unknown regulatory protein(s). The epimerase shows a surprising flexibility and, in addition to GDP-L-galactose, releases considerable amounts of GDP-L-gulose. The GDP-L-gulose-forming activity was rapidly lost within first hours after the enzyme purification, pointing to a partial inactivation of the epimerase (unpublished results). Unfortunately, most biochemical tests that involve the plant GDP-mannose 3″,5″-epimerase was conducted with a partially inactivated enzyme (Major et al., 2005; Linster et al., 2007). Unlike GDP-L-galactose but similarly to GDP-D-mannose, GDP-L-gulose is not a substrate for the VTC2 enzyme (Linster et al., 2007; Laing et al., 2007), probably because of the requirement of *trans*-configuration of the 2″- and 3″-hydroxyl groups of the GDP-bound hexose for the enzyme activity. Consequently, GDP-L-gulose does not enter the VTC2 cycle and, in the absence of an L-gulosyltransferase activity, would be dedicated for the synthesis of L-ascorbic acid via the L-gulose pathway. The L-gulose shunt could serve to escape some regulatory constraints of the VTC2 cycle, and to maintain the synthesis of vitamin C in photosynthetically-unfavorable conditions and in heterotrophic plant cells such as root-derived BY-2 cells of tobacco (Wolucka et al., 2005). Further studies will be necessary to elucidate the L-gulose pathway and the regulation of the GDP-L-gulose-releasing activity of the GDP-mannose 3″,5″-epimerase.

Although the GDP-mannose 2″-epimerase activity was suggested to exist in plants since a long time, the enzyme has not been identified and its physiological role is poorly understood. Identification and characterization of the plant GDP-D-mannose 2″-epimerase would be, therefore, a priority, and studies of protein-protein interactions in plants may offer a shortcut. The enzyme components of the proposed VTC2 cycle might operate as a complex, in which the VTC2 gyanylyltransferase physically interacts with the GDP-mannose 3″,5″-epimerase and, possibly, with an unknown GDP-Man 2″-epimerase. In the *de novo* pathway for GDP-L-fucose, the GDP-4″-keto-6″-deoxy-mannose 3″,5″-epimerase and the GDP-mannose 4″,6″-dehydratase form a complex that is necessary to stabilize the GDP-mannose 4″,6″-dehydratase enzyme activity

(Nakayama et al., 2003). In-depth studies of protein-protein interactions could help in understanding the regulation of the *de novo* biosynthesis of L-ascorbic acid in plants, but also facilitate the identification of GDP-mannose 2″-epimerase and, perhaps, of unknown regulatory proteins.

7. Conclusions

The non-inversion scheme of vitamin C biosynthesis (Loewus, 1963) and, later on, the identification of the first gene for vitamin C synthesis by the group of Marc Van Montagu (Ostergaard et al., 1997), the isolation of L-galactose dehydrogenase (Smirnoff and Wheeler, 1999) and the proposal of D-mannose pathway for vitamin C (Wheeler et al., 1998), followed by the generation of vitamin C-deficient mutant plants (Conklin et al., 1996,2000, the identification of GDP-mannose 3″,5″-epimerase (Wolucka et al., 2001) and other enzymes (Laing et al., 2004) and pathways (Agius et al., 2003; Wolucka and Van Montagu, 2003; Lorence et al., 2004), and, finally, the discovery of the formidable VTC2 engine for the *de novo* synthesis of vitamin C (Jander et al., 2002; Laing et al., 2007), have been the milestones in the race to the elucidation of vitamin C biosynthesis in plants.

However, there are still some unresolved issues that are important from the fundamental and biotechnological points of view, in particular, for generating transgenic plants with considerably increased vitamin C contents. One question is the identity of GDP-D-mannose 2''-epimerase and its possible role in the proposed here extended VTC2 cycle. Another point is the role of the GDP-L-gulose switch in the *de novo* synthesis of vitamin C, the identification of L-gulose specific enzymes, and the elucidation of molecular mechanisms of methyl jasmonate-dependent regulation of ascorbic acid synthesis. Moreover, in-depth studies of subcellular localization and protein-protein interactions, the determination of the role of the Hsc70.3 chaperone and other regulatory proteins, and further investigation of the physiological significance of D-galacturonate- and D-glucuronate-dependent salvage pathways, would help better understand the regulation of vitamin C synthesis in plants. Finally, the synthesis of L-ascorbic acid and analogs in bacteria and other microorganisms, its function in the interactions between pathogen/symbiont and the eukaryotic host, and potential biotechnological applications, is an emerging topic in the field of vitamin C research.

References

Agius, F., Gonzalez-Lamothe, R., Caballero, J.L., Munoz-Blanco, J., Botella, M.A., Valpuesta, V., 2003. Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat. Biotechnol. 21, 177–181.

Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S., Allen, A.E., Apt, K.E., Bechner, M., et al., 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306, 79–86.

Brenner, C., 2002. Hint, Fhit, and GalT: function, structure, evolution, and mechanism of three branches of the histidine triad superfamily of nucleotide hydrolases and transferases. Biochemistry 41, 9003–9014.

Conklin, P.L., Gatzek, S., Wheeler, G.L., Dowdle, J., Raymond, M.J., Rolinski, S., Isupov, M., Littlechild, J.A., Smirnoff, N., 2006. *Arabidopsis thaliana* VTC4 encodes L-galactose-1-P phosphatase, a plant ascorbic acid biosynthetic enzyme. J. Biol. Chem. 281, 15662–15670.

Conklin, P.L., Norris, S.R., Wheeler, G.L., Williams, E.H., Smirnoff, N., Last, R.L., 1999. Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. Proc. Natl. Acad. Sci. USA 96, 4198–4203.

Conklin, P.L., Saracco, S.A., Norris, S.R., Last, R.L., 2000. Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. Genetics 154, 847–856.

Conklin, P.L., Williams, E.H., Last, R.L., 1996. Environmental stress sensitivity of an ascorbic acid-deficient Arabidopsis mutant. Proc. Natl. Acad. Sci. USA 93, 9970–9974.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93.

Davies, M.B., Austin, J., Partridge, D.A., 1991. Vitamin C: Its Chemistry and Biochemistry. The Royal Society of Chemistry, Cambridge, UK.

Derelle, E., Ferraz, C., Rombauts, S., Rouze, P., Worden, A.Z., Robbens, S., Partensky, F., Degroeve, S., Echeynie, S., Cooke, R., et al., 2006. Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc. Natl. Acad. Sci. USA 103, 11647–11652.

Diaz de la Garza, R.I., Gregory III, J.F., Hanson, A.D., 2007. Folate biofortification of tomato fruit. Proc. Natl. Acad. Sci. USA 104, 4218–4222.

Elbein, A.D., 1969. Biosynthesis of a cell wall glucomannan in mung bean seedlings. J. Biol. Chem. 244, 1608–1616.

Everard, J.D., Cantini, C., Grumet, R., Plummer, J., Loescher, W.H., 1997. Molecular cloning of mannose-6-phosphate reductase and its developmental expression in celery. Plant Physiol. 113, 1427–1435.

Feingold, D.S., 1982. Aldo (and keto) hexoses and uronic acids. In: Loewus, F.A., Tanner, W. (Eds.), Plant Carbohydrates I. Intracellular Carbohydrates. Springer-Verlag, Berlin, pp. 3–76.

Fujiki, Y., Yoshikawa, Y., Sato, T., Inada, N., Ito, M., Nishida, I., Watanabe, A., 2001. Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. Physiol. Plant 111, 345–352.

Gao, Z., Xie, X., Ling, Y., Muthukrishnan, S., Liang, G.H., 2005. *Agrobacterium tumefaciens*-mediated sorghum transformation using a mannose selection system. Plant Biotechnol. J. 3, 591–599.

Gatzek, S., Wheeler, G.L., Smirnoff, N., 2002. Antisense suppression of l-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated l-galactose synthesis. Plant J. 30, 541–553.

Herold, A., Lewis, D.H., 1977. Mannose and green plants: occurrence, physiology and metabolism, and use as a tool to study the role of orthophosphate. New Phytol. 79, 1–40.

Imai, T., Kingston-Smith, A.H., Foyer, C.H., 1999. Ascorbate metabolism in potato leaves supplied with exogenous ascorbate. Free Radic. Res. 31 (Suppl), S171–S179.

Isherwood, F.A., Chen, Y.T., Mapson, L.W., 1953. Synthesis of l-ascorbic acid in plants and animals. Nature 171, 348–349.

Jain, A.K., Nessler, C.L., 2000. Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. Mol. Breed. 6, 73–78.

Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., Last, R.L., 2002. Arabidopsis map-based cloning in the post-genome era. Plant Physiol. 129, 440–450.

Laing, W.A., Bulley, S., Wright, M., Cooney, J., Jensen, D., Barraclough, D., MacRae, E., 2004. A highly specific L-galactose-1-phosphate

phosphatase on the path to ascorbate biosynthesis. Proc. Natl. Acad. Sci. USA 101, 16976–16981.

Laing, W.A., Wright, M.A., Cooney, J., Bulley, S.M., 2007. From the cover: the missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. Proc. Natl. Acad. Sci. USA 104, 9534–9539.

Liepman, A.H., Wilkerson, C.G., Keegstra, K., 2005. Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proc Natl Acad Sci U S A 102, 2221–2226.

Linster, C.L., Gomez, T.A., Christensen, K.C., Adler, L.N., Young, B.D., Brenner, C., Clarke, S.G., 2007. Arabidopsis VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J. Biol. Chem. 282, 18879–18885.

Loewus, F.A., 1963. Tracer studies on ascorbic acid formation in plants. Phytochemistry 2, 109–128.

Loewus, F.A., 1999. Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. Phytochemistry 52, 193–210.

Lorence, A., Chevone, B.I., Mendes, P., Nessler, C.L., 2004. Myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant Physiol. 134, 1200–1205.

Major, L.L., Wolucka, B.A., Naismith, J.H., 2005. Structure and function of GDP-mannose-3′,5′-epimerase: an enzyme which performs three chemical reactions at the same active site. J. Am. Chem. Soc. 127, 18309–18320.

Matsuzaki, M., Misumi, O., Shin, I.T., Maruyama, S., Takahara, M., Miyagishima, S.Y., Mori, T., Nishida, K., Yagisawa, F., Yoshida, Y., et al., 2004. Genome sequence of the ultrasmall unicellular red alga CYANIDIOSCHYZON MEROLAE 10D. Nature 428, 653–657.

Mengele, R., Sumper, M., 1992. Gulose as a constituent of a glycoprotein. FEBS Lett. 298, 14–16.

Nakayama, K., Maeda, Y., Jigami, Y., 2003. Interaction of GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase with GDP-mannose-4,6-dehydratase stabilizes the enzyme activity for formation of GDP-fucose from GDP-mannose. Glycobiology 13, 673–680.

Olmos, E., Kiddle, G., Pellny, T., Kumar, S., Foyer, C., 2006. Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. J. Exp. Bot. 57, 1645–1655.

Ostergaard, J., Persiau, G., Davey, M.W., Bauw, G., Van Montagu, M., 1997. Isolation of a cDNA coding for L-galactono-gamma-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. Purification, characterization, cDNA cloning, and expression in yeast. J. Biol. Chem. 272, 30009–30016.

Privalle, L.S., 2002. Phosphomannose isomerase, a novel plant selection system: potential allergenicity assessment. Ann. NY. Acad. Sci. 964, 129–138.

Qian, W., Yu, C., Qin, H., Liu, X., Zhang, A., Johansen, I.E., Wang, D., 2007. Molecular and functional analysis of phosphomannomutase (PMM) from higher plants and genetic evidence for the involvement of PMM in ascorbic acid biosynthesis in *Arabidopsis* and *Nicotiana benthamiana*. Plant J. 49, 399–413.

Rumpho, M.E., Edwards, G.E., Loescher, W.H., 1983. A pathway for photosynthetic carbon flow to mannitol in celery leaves : activity and localization of key enzymes. Plant Physiol. 73, 869–873.

Running, J.A., Burlingame, R.P., Berry, A., 2003. The pathway of L-ascorbic acid biosynthesis in the colourless microalga *Prototheca moriformis*. J. Exp. Bot. 54, 1841–1849.

Schiermeier, Q., 2000. Novartis pins hopes for GM seeds on new marker system. Nature 406, 924.

Shintani, D., DellaPenna, D., 1998. Elevating the vitamin E content of plants through metabolic engineering. Science 282, 2098–2100.

Smirnoff, N., Wheeler, G., 1999. Plant galactose dehydrogenase. World Intellectual Property Organization Patent No. WO 9933995.

Stein, J.C., Hansen, G., 1999. Mannose induces an endonuclease responsible for DNA laddering in plant cells. Plant Physiol. 121, 71–80.

Watanabe, K., Suzuki, K., Kitamura, S., 2006. Characterization of a GDP-D-mannose 3″,5″-epimerase from rice. Phytochemistry 67, 338–346.

Watkins, C.B., Frenkel, C., 1987. Inhibition of pear fruit ripening by mannose. Plant Physiol. 85, 56–61.

Wenck, A., Hansen, G., 2005. Positive selection. Meth. Mol. Biol. 286, 227–236.

Wheeler, G.L., Jones, M.A., Smirnoff, N., 1998. The biosynthetic pathway of vitamin C in higher plants. Nature 393, 365–369.

Wolucka, B., 2005. L-Gulose dependent vitamin C synthesis. World Intellectual Property Organization Patent No. WO 2005014844.

Wolucka, B.A., Communi, D., 2006. Mycobacterium tuberculosis possesses a functional enzyme for the synthesis of vitamin C, L-gulono-1,4-lactone dehydrogenase. Febs. J. 273, 4435–4445.

Wolucka, B.A., Goossens, A., Inze, D., 2005. Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. J. Exp. Bot. 56, 2527–2538.

Wolucka, B.A., Persiau, G., Van Doorsselaere, J., Davey, M.W., Demol, H., Vandekerckhove, J., Van Montagu, M., Zabeau, M., Boerjan, W., 2001. Partial purification and identification of GDP-mannose 3″,5″-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway. Proc. Natl. Acad. Sci. USA 98, 14843–14848.

Wolucka, B.A., Van Montagu, M., 2003. GDP-mannose 3′,5′-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosynthesis of vitamin C in plants. J. Biol. Chem. 278, 47483–47490.

Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I., 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287, 303–305.

Zhu, Y.J., Agbayani, R., McCafferty, H., Albert, H.H., Moore, P.H., 2005. Effective selection of transgenic papaya plants with the PMI/Man selection system. Plant Cell Rep. 24, 426–432.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., Gruissem, W., 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. 136, 2621–2632.