Opinion

L-Ascorbate biosynthesis in higher plants: the role of VTC2

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In the past year, the last missing enzyme of the L-galactose pathway, the linear form of which appears to represent the major biosynthetic route to L-ascorbate (vitamin C) in higher plants, has been identified as a GDP-L-galactose phosphorylase. This enzyme catalyzes the first committed step in the synthesis of that vital antioxidant and enzyme cofactor. Here, we discuss how GDP-L-galactose phosphorylase enzymes, encoded in *Arabidopsis* by the paralogous *VTC2* and *VTC5* genes, function in concert with the other enzymes of the L-galactose pathway to provide plants with the appropriate levels of L-ascorbate. We hypothesize that regulation of L-ascorbate biosynthesis might occur at more than one step and warrants further investigation to allow for the manipulation of vitamin C levels in plants.

Pathways to L-ascorbate in plants

L-Ascorbate (or vitamin C) is an essential enzyme cofactor in hydroxylation and other reactions, as well as a primary antioxidant in both plants and animals [1]. Because a few animal species (including primates) have lost the capacity for L-ascorbate synthesis [2], they are dependent upon diet to ensure adequate levels for metabolism and oxidative protection. The high L-ascorbate contents found in plants (which have an average cellular L-ascorbate concentration of 2–25 mM or more in the chloroplast [3]) make them the primary source of vitamin C intake for humans. In plants, L-ascorbate has been implicated in processes including growth [4], programmed cell death [5], pathogen responses [6], hormone responses, flowering and senescence [7], as well as protection against environmental stresses including ozone [8], UV radiation [9], high temperatures [10] and high light intensity [11].

Understanding the biosynthetic pathway(s) for L-ascorbate in plant cells is an important aspect in the effort to elucidate how L-ascorbate levels are maintained in these cells. Surprisingly, it is only recently that the major plant pathway, which is different from the animal L-ascorbate synthesis pathway [2], has been described. It was not until 1998 that, based on tracer studies and biochemical analyses, a pathway (the L-galactose pathway) encompassing a series of earlier observations was proposed [12]. This pathway involves ten enzymatic steps from D-glucose to L-ascorbate via the intermediate formation of GDP-D-mannose and L-galactose (Figure 1). Although alternative pathways have been suggested [13–15], specific enzymes and the corresponding genes have not yet been identified for most of the proposed reactions (Figure 1). In the last few years, a combination of genetic and biochemical approaches has demonstrated that the L-galactose pathway is the dominant biosynthetic route to L-ascorbate in many plants and has allowed us to begin to understand the regulation of the pathway. With the recent characterization of the *VTC2* and *VTC5* expression products as GDP-L-galactose phosphorylases [16–19], genes have now been identified for all of the reactions involved in this pathway.

The first six steps of the L-galactose pathway are used to synthesize activated nucleotide sugars that are also precursors of cell wall polysaccharides and glycoproteins (Figure 1). The committed pathway to L-ascorbate biosynthesis then consists of the sequential conversion of GDP-L-galactose into L-galactose-1-P, L-galactose, L-galactono-1,4-lactone, and L-ascorbate. Since their discovery in 2007, there has been great interest in the enzymes (VTC2 and VTC5) catalyzing the first reaction of the committed pathway. Here, we discuss this step and the controversies surrounding it after briefly reviewing recent work concerning the other reactions of the L-galactose pathway. On the basis of these studies, we propose that regulation of L-ascorbate synthesis in plants is complex, occurring at the level of GDP-L-galactose phosphorylase and at possible additional steps.

Activities and regulation of the L-galactose pathway enzymes

The initial three steps to D-mannose-6-P – an essential part of the pathway?

Whereas hexokinase and phosphoglucose isomerase are well-established players in glycolysis, phosphomannose isomerase (PMI), the enzyme forming D-mannose-6-P, has not been extensively studied. Based on sequence homology, two putative *PMI* genes can be found in the *Arabidopsis thaliana* genome, At3g02570 and At1g67070. Little is known about the former gene, but transcripts of the latter gene (also named *din9*) could be detected in leaves only 24 h after dark treatment [20]; biochemical and genetic evidence has been presented that PMI is either not present or occurs at low levels in plants [21,22]. These observations question the occurrence of the L-galactose pathway as currently proposed (Figure 1) and have prompted Wolucka and Van Montagu [23] to propose the VTC2 cycle described in the next section and in Figure 2b. However, the lower L-ascorbate contents observed in plants with decreased phosphomannomutase (PMM) transcript levels [24] and in plants deficient in GDP-D-mannose pyr-

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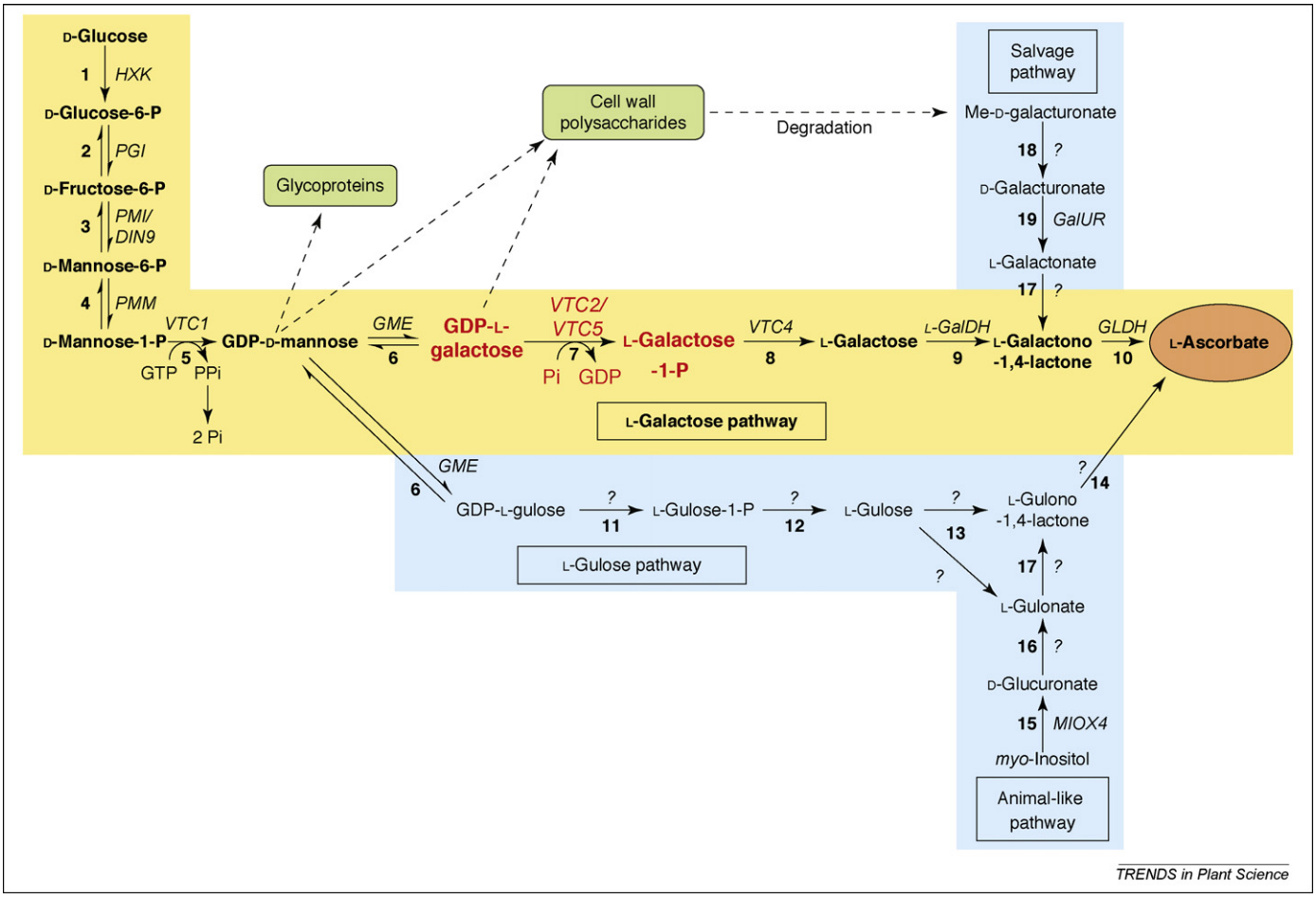

Figure 1. Biosynthetic routes to L-ascorbate in higher plants. The major L-galactose pathway is highlighted in yellow with intermediates in bold type [12]; alternative proposed biosynthetic routes are highlighted in blue [13–15]. Designations are given in italics for genes encoding known enzymes of the pathways; question marks indicate possible reactions where the gene and the specific enzyme have not yet been identified. The central reaction (and the first committed step for L-ascorbate synthesis) in the L-galactose pathway, which is catalyzed by VTC2 and VTC5, is shown in red. Enzymes catalyzing the numbered reactions are: 1, hexokinase; 2, phosphoglucose isomerase; 3, phosphomannose isomerase (PMI); 4, phosphomannomutase (PMM); 5, GDP-D-mannose pyrophosphorylase; 6, GDP-D-mannose 3′,5′-epimerase (GME); 7, GDP-L-galactose phosphorylase; 8, L-galactose-1-P phosphatase; 9, L-galactose dehydrogenase; 10, L-galactono-1,4-lactone dehydrogenase (GLDH); 11, nucleotide pyrophosphatase or sugar-1-P guanylyltransferase; 12, sugar phosphatase; 13, sugar dehydrogenase; 14, L-gulono-1,4-lactone dehydrogenase/oxidase; 15, *myo*-inositol oxygenase; 16, uronate reductase; 17, aldonolactonase; 18, methylesterase; 19, D-galacturonate reductase.

ophosphorylase activity [25] argue in favor of the involvement of D-mannose-6-P formation in L-ascorbate synthesis. Moreover, PMI activity has been recently demonstrated in *Arabidopsis* leaf extracts [18], and strong evidence for the *in vivo* involvement of this activity in GDP-D-mannose synthesis has been provided using a novel dual-radiolabeling strategy in *Arabidopsis* cell cultures [26]. Although the gene(s) responsible for PMI activity in *Arabidopsis* still need(s) to be characterized, these recent observations support the idea that PMI activity is present in plants.

PMM and GDP-D-mannose pyrophosphorylase (VTC1) – regulatory sites for L-ascorbate synthesis?

The activities of the next two enzymes in the L-galactose pathway (PMM and VTC1) result in the formation of GDP-D-mannose (Figure 1). L-Ascorbate contents in *Nicotiana benthamiana* and/or *Arabidopsis* are modulated by alteration of *PMM* transcript levels through virus-induced gene silencing and transgenic expression [24], as well as by *PMM* mutation [27]. These observations suggest that PMM activity might affect the overall rate of L-ascorbate biosynthesis.

An ozone-sensitive *Arabidopsis* mutant accumulating only 30% of the normal L-ascorbate concentration [28] has been mapped to a locus (*VTC1*) encoding a GDP-D-mannose pyrophosphorylase [25]. Antisense inhibition of this enzyme in potato also resulted in lowered L-ascorbate levels [29]. Indeed, mRNA levels of GDP-mannose pyrophosphorylase are correlated with L-ascorbate levels in several plant species [30,31]. Finally, jasmonates, which have been shown to increase L-ascorbate levels in *Arabidopsis*, were also found to induce the expression of *VTC1* in this organism [32,33]. Taken together, these results suggest that some control of L-ascorbate synthesis could occur at this step. However, since GDP-D-mannose is also required for cell wall biogenesis and protein glycosylation [34], crucial regulatory control might be expected to occur elsewhere.

GDP-D-mannose 3′,5′-epimerase (GME) – an initiator of a new branch for L-ascorbate biosynthesis?

GME converts GDP-D-mannose not only to GDP-L-galactose, but also to GDP-L-gulose (the C5′ epimer of GDP-D-mannose) [35,36]. The latter has been proposed to initiate an alternative branch (the L-gulose pathway; see Figure 1) for *de novo* L-ascorbate biosynthesis in plants [35]. The modulation of GME activity by several metabolites [35,36] and the induction of *GME* expression by methyl jasmonate

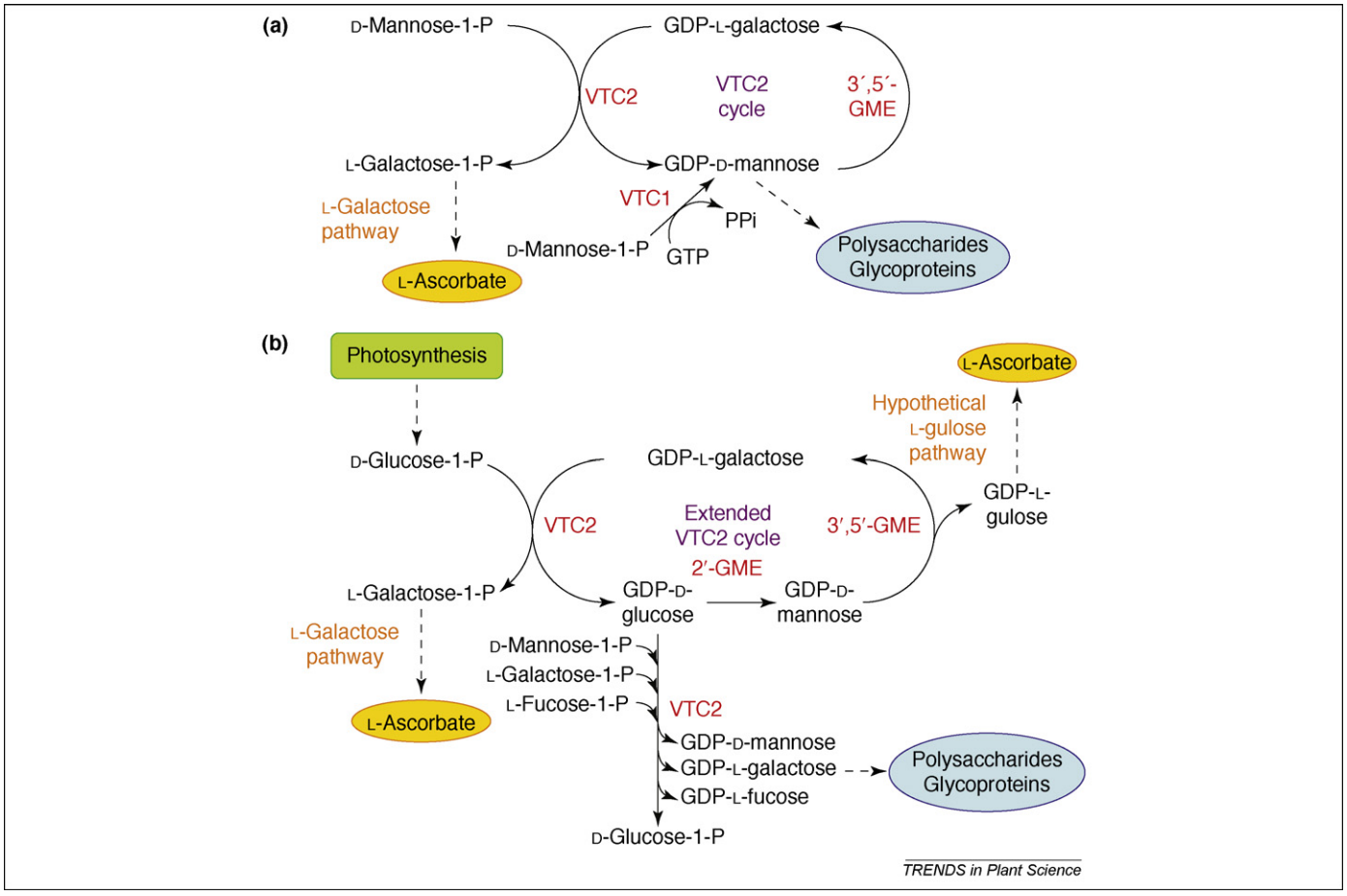

Figure 2. Proposed VTC2 cycles. **(a)** The original VTC2 cycle proposed by Laing *et al.* [17] requires only two enzymes, VTC2 and GDP-D-mannose 3′,5′-epimerase (3′,5′-GME), to generate L-galactose-1-P from D-mannose-1-P for L-ascorbate biosynthesis. In this scheme, GDP-D-mannose generated by the transferase activity of VTC2 is recycled to the GDP-L-galactose substrate of VTC2 by 3′,5′-GME. VTC1 activity is only required to compensate for the GDP-D-mannose molecules that are drained from the cycle for polysaccharide and glycoprotein synthesis. **(b)** The extended VTC2 cycle proposed by Wolucka and Van Montagu [23] involves an additional putative enzyme that interconverts GDP-D-glucose and GDP-D-mannose (2′-GME). Here, D-glucose-1-P, derived from photosynthesis, is converted to L-galactose-1-P by the VTC2 transferase activity. L-Galactose-1-P is used for L-ascorbate synthesis; the other VTC2 product (GDP-D-glucose) can be converted to other GDP-hexoses for polysaccharide and glycoprotein synthesis or can be recycled to GDP-L-galactose through the successive action of the putative 2′-GME and 3′,5′-GME. The priming reaction to start this VTC2 cycle could involve a GDP-D-glucose pyrophosphorylase or VTC1 (not shown). Given the low transferase activities measured with VTC2 and the hypothetical nature of the 2′-GME activity, further work is needed to determine whether either of these cycles is physiologically relevant in plants.

in tobacco cells [33] suggest that this step might be a control point in L-ascorbate biosynthesis.

GDP-L-galactose phosphorylase (VTC2, VTC5) – the first committed step in L-ascorbate biosynthesis

The enzyme converting GDP-L-galactose to L-galactose-1-P has only been identified very recently; this step appears to have a major role in the regulation of L-ascorbate synthesis, and we will return to focus on it after discussion of the remaining reactions of the pathway.

L-Galactose-1-P phosphatase (VTC4)

VTC4, initially identified from an L-ascorbate-deficient *Arabidopsis* mutant [37], encodes a specific L-galactose-1-P phosphatase [38,39]. However, the observation that a VTC4-knockout mutant is only partially deficient in L-ascorbate as well as L-galactose-1-P phosphatase activity suggests that VTC4 is not the only enzyme catalyzing this reaction in *Arabidopsis* [39]. The purple acid phosphatase AtPAP15, whose overexpression has recently been shown to increase foliar L-ascorbate in *Arabidopsis* [40], might contribute to the hydrolysis of L-galactose-1-P; the latter has, however, not yet been tested as a substrate. Although *VTC4* mRNA levels have been correlated with changes in L-ascorbate levels in response to light [41], no change in enzymatic activity was found under such conditions [18].

L-Galactose dehydrogenase – a target for feedback inhibition?

Spinach L-galactose dehydrogenase exhibits reversible competitive inhibition by L-ascorbate with a K_i of 0.13 mM [42], thus making it a possible target for the reported end-product feedback inhibition of L-ascorbate biosynthesis [43]. However, the substantial accumulation of L-ascorbate observed after L-galactose feeding [12] and the lack of effect of *Arabidopsis* L-galactose dehydrogenase overexpression on L-ascorbate contents in tobacco [44] suggest that this inhibition is absent or attenuated *in vivo* and that L-galactose dehydrogenase exerts little control over flux through the L-galactose pathway.

L-Galactono-1,4-lactone dehydrogenase (GLDH) – in a favorable position for regulation by respiration?

The last step of L-ascorbate biosynthesis is catalyzed by a mitochondrial flavin-containing dehydrogenase that is highly specific for L-galactono-1,4-lactone and uses cyto-

chrome *c* as an electron acceptor in the respiratory chain [13,45,46]. The transcript level and enzyme activity of GLDH have been observed to positively correlate with L-ascorbate content in various plant tissues, although there is species-to-species variation (reviewed in [14]). However, reduction of GLDH activity by RNA interference in tomato, which led to decreased growth rates, did not alter the L-ascorbate synthesis capacity in the transgenic plants [47]. It should be noted that, based on several observations, the existence of different isoforms of GLDH, some of which might use L-gulono-1,4-lactone, has been suggested [45,46]. GLDH seems to have a role in the control of L-ascorbate content by light [18,41,48]; the mechanism is unclear but might involve light-dependent regulation of *GLDH* expression [49], as well as light-dependent changes in respiration that might directly affect GLDH activity [50,51].

GDP-L-galactose phosphorylase – a central player in plant L-ascorbate biosynthesis?

The first metabolite in the pathway dedicated solely to L-ascorbate biosynthesis is L-galactose-1-P; the enzyme catalyzing its formation thus represents the committed step in the overall pathway. As such, this enzyme has been a focus of intense recent study.

Identification of VTC2 as the enzyme converting GDP-L-galactose to L-galactose-1-P and controversy surrounding its activity

The identity of this enzyme eluded researchers until 2007. Three vitamin-C-deficient *Arabidopsis* mutants were mapped to a locus on chromosome 4 that was designated *VTC2* (vitamin C 2) [37]. These mutations were then located in the uncharacterized At4g26850 gene [52]. BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) analyses of the predicted amino acid sequence of VTC2 revealed the existence of a motif characteristic of the histidine triad (HIT) protein superfamily [16–18], whose members are hydrolases, phosphorylases or transferases acting on nucleotide-containing substrates [53]. The conversion of GDP-L-galactose to L-galactose-1-P in the L-galactose pathway represents such a type of reaction and suggested that VTC2 could be the long-sought-after enzyme forming L-galactose-1-P. All known HIT enzymes use the second His residue (His238 in *Arabidopsis* VTC2) of the HIT motif to attack the α-phosphate of the monophosphonucleoside moiety of their substrates, leading to covalent nucleotidylylation of this His residue [53]. The nucleotidylylated enzyme intermediate can then be broken down either by simple hydrolysis or by nucleotidylyl transfer to inorganic phosphate (phosphorolysis) or a specific phosphorylated compound (transfer). In the case of VTC2, breakdown of the putative guanylylated enzyme intermediate formed could thus in theory proceed in the absence of a specific guanylyl acceptor (hydrolysis) or require the presence of inorganic phosphate or a phosphorylated compound.

In three independent studies, *Escherichia coli*-expressed recombinant *Arabidopsis* VTC2 [16,18] or a kiwifruit homolog thereof [17] were found to specifically convert GDP-L-galactose to L-galactose-1-P and to require the presence of a guanylyl acceptor other than water to catalyze the reaction. Moreover, evidence for covalent guanylylation of the second His residue of the HIT motif of *Arabidopsis* VTC2 has been provided [19]. These results established VTC2 as a novel member of the phosphorylase-transferase (but not hydrolase) branch of the HIT protein

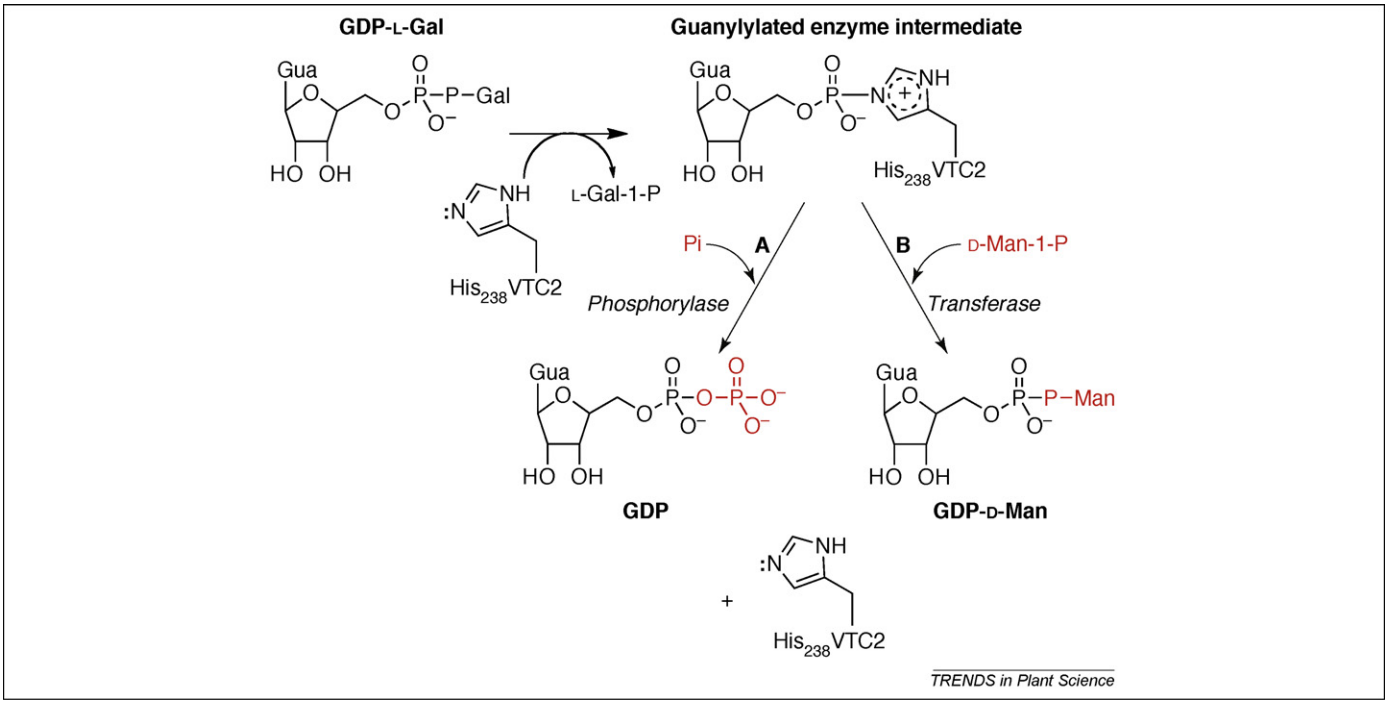

Figure 3. Proposed VTC2-catalyzed reactions. The reaction catalyzed by VTC2 proceeds in two steps. In the first step, VTC2 forms a covalent guanylylated active site His intermediate with the α-phosphate of GDP-L-galactose, releasing L-galactose-1-P. In the second step, the enzyme could alternatively transfer the guanylyl group to inorganic phosphate, leading to a phosphorylase activity generating GDP (pathway A), or to a hexose-1-P, leading to a transferase activity generating a GDP-hexose (pathway B). Pathway A appears to be the major route in higher plants because the phosphorylase activity of VTC2 was found to be more than 100-fold higher than its transferase activity (see the main text).

superfamily and showed that this enzyme [0]catalyzes the last unidentified step of the L-galactose pathway.

However, there is some controversy [23] over whether the physiological guanylyl acceptor is inorganic phosphate, leading to a phosphorylase activity that generates GDP [16,18], or a hexose-1-P, leading to a transferase activity that generates the corresponding GDP-hexose [17] (Figure 3). This is an important issue that impacts the feasibility of the VTC2 cycles that have been proposed recently as an alternative to the linear L-galactose pathway allowing maintenance of high-energy phosphate bonds during L-ascorbate synthesis (Figure 2 and see later in this section). Linster *et al.* [16] and Dowdle *et al.* [18] found high VTC2 activities in the presence of inorganic phosphate and characterized the enzyme as a GDP-L-galactose phosphorylase; Laing *et al.* [17] found higher catalytic efficiencies when phosphate was replaced by hexose-1-phosphates, such as D-mannose-1-P, D-glucose-1-P, D-galactose-1-P or L-*myo*-inositol-1-P, and thus described the enzyme as a GDP-L-galactose-hexose-1-P guanylyltransferase. However, a subsequent analysis of the activity of *Arabidopsis* VTC2 in the presence of different guanylyl acceptors using a direct high performance liquid chromatography (HPLC)-based assay [19] failed to confirm the results obtained by Laing *et al.* [17], who utilized an indirect enzyme-coupled assay. On the contrary, VTC2 was found to be more than 100-fold more efficient as a GDP-L-galactose phosphorylase than as a GDP-L-galactose-D-glucose-1-P guanylyltransferase [19]. Phosphate was also preferred over D-glucose-1-P when partially purified extracts of *Arabidopsis*, Japanese mustard spinach, lemon, spinach and maize were used as the enzyme source [19]. Moreover, using the HPLC-based assay, conversion of GDP-L-galactose to L-galactose-1-P in the presence of D-mannose-1-P (the compound proposed by Laing *et al.* [17] to be the endogenous guanylyl acceptor of VTC2) could be measured neither with recombinant VTC2 nor with partially purified plant extracts [19]. Taken together, these results suggest that in most plant species, VTC2 acts predominantly as a phosphorylase.

Alternative reactions catalyzed by VTC2

Interestingly, VTC2 does not display absolute specificity for its nucleotide sugar substrate; similar catalytic efficiencies were found with GDP-L-galactose and with GDP-D-glucose [16]. Significant phosphorylase activity could also be measured with GDP-L-fucose [19], whereas GDP-D-mannose turned out to be a very poor substrate [16]. No activity was detected in the presence of GDP-L-gulose, a series of UDP-sugars, and ADP-D-glucose [16,18]. VTC2 is thus more specific for the nucleotide moiety than for the sugar moiety of the nucleotide sugar substrate. This specificity (or lack of) is important in formulating the VTC2 cycles shown in Figure 2 and discussed later in this section.

VTC5: a second GDP-L-galactose phosphorylase in Arabidopsis

The *VTC2-2* and *VTC2-3* mutant alleles resulting in L-ascorbate deficiency lead to G224D and S290F substitutions, respectively [18,52]. No residual activity was detected with recombinant VTC2 containing the aspartate residue at position 224 [18,19]. Compared with the wild-type enzyme, the recombinant VTC2 protein containing the phenylalanine residue at position 290 displayed a higher K_m for inorganic phosphate and a lower k_{cat}, leading to a 50-fold decrease in the catalytic efficiency [18]. The relatively high residual L-ascorbate contents (25–50% of wild-type levels) found in these mutants [18] thus suggested the existence of VTC2-independent L-ascorbate biosynthesis pathways and/or of other enzymes catalyzing a VTC2-like reaction.

In fact, a gene (At5g55120) sharing high sequence identity with *VTC2* was identified in the *Arabidopsis* genome, and its expression product, designated VTC5, was found to be a GDP-L-galactose phosphorylase whose kinetic properties greatly resemble those of VTC2 [18,19]. Similarly to VTC2, VTC5 is much more efficient as a GDP-L-galactose phosphorylase than as a GDP-L-galactose-hexose-1-P guanylyltransferase [19]. Both *VTC2* and *VTC5* are expressed in leaf, stem, root, flower and silique tissue, but the expression level of *VTC5* is generally 100- to 1000-times lower than that of *VTC2* [18]. Accordingly, the leaf L-ascorbate contents of two homozygous T-DNA insertion mutants (*vtc5-1* and *vtc5-2*) lacking *VTC5* transcripts were not markedly different from those found in wild-type plants [18].

Significantly, *vtc2 vtc5* double-mutant seedlings stopped growing after initial expansion of the cotyledons, which then bleached within two weeks [18]. These seedlings could, however, be rescued by supplementation with L-galactose or L-ascorbate. Larger plants rescued on L-galactose showed bleaching of older leaves one week after transfer to L-galactose-free medium. These results show that the GDP-L-galactose phosphorylase activities of VTC2 and VTC5 are required for seedling viability and probably also at later growth stages. Importantly, they also demonstrate that the L-galactose pathway is the only significant source of L-ascorbate in *Arabidopsis* seedlings.

Regulation of VTC2 and VTC5 activities

Because GDP-D-mannose and GDP-L-galactose are not only used for L-ascorbate formation but also in the synthesis of cell wall polysaccharides and/or protein glycosylation [21,54], the phosphorylase reaction is the first committed step in the L-galactose pathway and thus VTC2 and VTC5 are good potential targets for the regulation of L-ascorbate synthesis. L-Ascorbate, L-galactono-1,4-lactone and L-galactose had no effect on VTC2 activity, indicating no feedback regulation of the enzyme by these metabolites [18]. However, L-ascorbate supplementation decreased *VTC2* expression in *Arabidopsis* plants, suggesting feedback inhibition by L-ascorbate at the transcriptional level [18]. The increase in leaf L-ascorbate content measured after a 24 h exposure to high light in *Arabidopsis* was accompanied by increased expression of *VTC2* and *VTC5* and by a 20-fold increase in GDP-L-galactose phosphorylase activity [18]. Light induction of *VTC2* mRNA has been confirmed in two additional independent studies [41,55]. However, except for a small (two-fold) increase in L-galactono-1,4-lactone dehydrogenase activity, none of the other enzymes of the L-galactose pathway were found to be affected by acclimation to high light

[18]. Evidence has been presented that increased L-galactose synthesis, involving a step upstream of L-galactose dehydrogenase, contributes to the light-induced accumulation of L-ascorbate [44]; it now seems to be very likely that this accumulation is largely mediated by increased VTC2 and/or VTC5 activity. In addition to the response to light intensity, *VTC2* and *VTC5* expression might also be under the control of the circadian clock [18]. Furthermore, jasmonates induced *VTC2* and *VTC5* transcription, in addition to *VTC1* transcription, in *Arabidopsis* [32]. Taken together, these observations suggest that regulation of *VTC2* and *VTC5* expression has a major role in controlling L-ascorbate biosynthesis. This is further supported by the finding that transient overexpression of the kiwifruit homolog of *VTC2* in tobacco leaves led to a threefold increase in L-ascorbate content, indicating that this enzyme is rate-limiting for L-ascorbate synthesis [17]. Preliminary evidence for a nuclear localization, in addition to its cytosolic localization, has been provided for the VTC2 protein, suggesting a potential nuclear function for VTC2 [55]; the intracellular localization of VTC5 was not analyzed in this study.

Existence of a VTC2 cycle?

Based on the D-mannose-1-P guanylyltransferase activity they measure, Laing *et al.* [17] proposed a VTC2 cycle in which the biosynthesis of L-galactose-1-P could be sustained by the action of VTC2 and GME in the absence of VTC1 (Figure 2a), thus avoiding the net hydrolysis of GTP to GDP and inorganic phosphate (Figure 1). In such a cycle, VTC1 is only required to resupply GDP-D-mannose that is taken from the cycle by the reactions leading to polysaccharide and glycoprotein formation.

Wolucka and Van Montagu [23] proposed an extended VTC2 cycle (Figure 2b) that could in addition account for L-ascorbate synthesis in the absence of PMI activity (see previous section). This extended cycle is also dependent upon the use of hexose-phosphates as guanylyl acceptors by VTC2. Additionally, it requires the presence of an as yet unidentified 2′-epimerase that interconverts GDP-D-glucose and GDP-D-mannose (Figure 2b). In this scheme, the products of photosynthesis could directly enter the extended VTC2 cycle with no need for PMI activity. This cycle would generate, in addition to L-galactose-1-P used for L-ascorbate production, GDP-hexoses for polysaccharide and glycoprotein synthesis and conserve energy by preserving the phosphodiester linkage in the GDP-hexoses.

However, the failure to confirm the initially reported transferase activity of VTC2 [17] in both VTC2 and VTC5 [19] represents an obstacle to the physiological operation of both types of cycles in plants. Furthermore, the recent dual-radiolabeling study described in the previous section [26] provides convincing evidence that, in *Arabidopsis* cells, GDP-D-mannose is synthesized predominantly via a pathway involving PMI rather than through a pathway involving GDP-D-glucose epimerization. Clearly, further work is needed to experimentally demonstrate the postulated GDP-D-mannose 2′-epimerase activity and to determine whether hexose-phosphates can be guanylyl acceptors for endogenous VTC2 and/or VTC5 enzymes that might be covalently modified, allosterically regulated or complexed with other proteins.

Concluding remarks

Control of L-ascorbate steady-state levels in plants potentially involves regulation of biosynthesis, catabolism, recycling and transport of this compound. Only regulation of biosynthesis is discussed here; current knowledge about catabolism, recycling and transport of L-ascorbate has recently been reviewed by others [13,14,56]. Although several enzymes of the L-galactose pathway present regulatory aspects, it seems that major control of L-ascorbate synthesis occurs at the step catalyzed by VTC2 and VTC5. Further experimental work is required to understand the mechanisms of this control, including how *VTC2* and *VTC5* expression is regulated at transcriptional, post-transcriptional and translational levels and how their enzymatic activities are potentially modulated by covalent modification, allosteric regulation and interaction with other proteins. Additionally, studies will be needed to verify the participation of other steps in the control of this pathway, as well as to clarify the role of the alternative L-ascorbate biosynthesis pathways that have been proposed. Understanding the regulatory mechanisms could then allow for testing genetic and/or small molecule approaches for manipulating plant L-ascorbate levels. Such intervention could generate plants with increased resistance to oxidative stress, as well as fruits and vegetables with longer shelf life and higher nutritional value for humans.

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