Arabidopsis *VTC2* Encodes a GDP-L-Galactose Phosphorylase, the Last Unknown Enzyme in the Smirnoff-Wheeler Pathway to Ascorbic Acid in Plants[*][♦]

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The first committed step in the biosynthesis of L-ascorbate from D-glucose in plants requires conversion of GDP-L-galactose to L-galactose 1-phosphate by a previously unidentified enzyme. Here we show that the protein encoded by *VTC2*, a gene mutated in vitamin C-deficient *Arabidopsis thaliana* strains, is a member of the GalT/Apa1 branch of the histidine triad protein superfamily that catalyzes the conversion of GDP-L-galactose to L-galactose 1-phosphate in a reaction that consumes inorganic phosphate and produces GDP. In characterizing recombinant VTC2 from *A. thaliana* as a specific GDP-L-galactose/GDP-D-glucose phosphorylase, we conclude that enzymes catalyzing each of the ten steps of the Smirnoff-Wheeler pathway from glucose to ascorbate have been identified. Finally, we identify VTC2 homologs in plants, invertebrates, and vertebrates, suggesting that a similar reaction is used widely in nature.

Vitamin C (L-ascorbic acid) is well known as an important antioxidant and enzyme cofactor in animals (1, 2) and in plants (3). Apparently, all plants are able to produce vitamin C and mutants completely deficient in synthesis have not been described, suggesting an essential role of ascorbate biosynthesis in these organisms (4). Although vertebrate vitamin C synthesis is restricted to one organ (liver in mammals and kidney in fish, amphibians, and reptiles) (5, 6), virtually all cells in plants can form ascorbate (4). In the few vertebrate species, such as humans, which lack ascorbate biosynthesis, loss of the pathway is compensated by dietary intake, particularly from plants.

Different pathways of ascorbate synthesis have evolved in animals and plants. In animals, ascorbate is formed from UDP-D-glucuronate in a pathway involving D-glucuronate formation, reduction and lactonization of D-glucuronate to L-gulonolactone and oxidation of the latter to L-ascorbate (reviewed in Ref. 7). Deficiency of the enzyme catalyzing this last step (L-gulonolactone oxidase) is responsible for the loss of ascorbate synthesis in the vitamin C-requiring vertebrates (8). In plants, the ascorbate synthesis pathway has remained elusive until recently and alternative pathways may exist (9). The Smirnoff-Wheeler pathway (10) has garnered strong biochemical and genetic support (11–16) and appears to represent the major route to ascorbate biosynthesis. In this pathway, GDP-D-mannose, formed from D-mannose 1-phosphate, is successively converted to GDP-L-galactose, L-galactose 1-phosphate, L-galactose, L-galactono-1,4-lactone, and finally to L-ascorbate.

Screens for ozone-sensitive (17) or ascorbate-deficient (18) mutants in *Arabidopsis thaliana* led to the identification of four loci (*VTC1*, *VTC2*, *VTC3*, and *VTC4*) involved in the maintenance of the vitamin C pool. Characterization of the *vtc1* (19) and *vtc4* (15) mutants, as well as biochemical studies (14), have allowed the identification of two of the enzymes required for L-ascorbic acid synthesis through the Smirnoff-Wheeler pathway. *VTC1* and *VTC4* encode GDP-mannose pyrophosphorylase (19) and L-Gal-1-P[4] phosphatase (15), respectively. The function of the *VTC2* and *VTC3* genes has not yet been elucidated. The only step in the Smirnoff-Wheeler pathway that has not been identified with a gene product is the conversion of GDP-L-Gal to L-Gal-1-P.

Here we show that the product of the *A. thaliana VTC2* gene, which has previously been cloned (20), is broadly conserved in plants and in animals. On the basis of sequence similarity and enzymatic analysis, we characterize VTC2 as a histidine triad (HIT) enzyme of the GalT/Apa1 branch possessing a high GDP-L-Gal phosphorylase activity, which produces GDP and the ascorbic acid precursor L-Gal-1-P. This finding completes the identification of the ten enzymatic steps from D-glucose to L-ascorbate in the Smirnoff-Wheeler pathway for vitamin C biosynthesis in plants.

EXPERIMENTAL PROCEDURES

Materials—ADP-D-Glc, GDP, GDP-D-Glc, GDP-D-Man, UDP-D-Gal, UDP-D-Glc, D-Gal-1-P, and D-Man-1-P were from

[4] The abbreviations used are: Gal-1-P, galactose 1-phosphate; GDP-L-Gul, GDP-L-gulose; Glc-1-P, glucose 1-phosphate; GME, GDP-D-mannose 3',5'-epimerase; HIT, histidine triad; Man-1-P, mannose 1-phosphate.
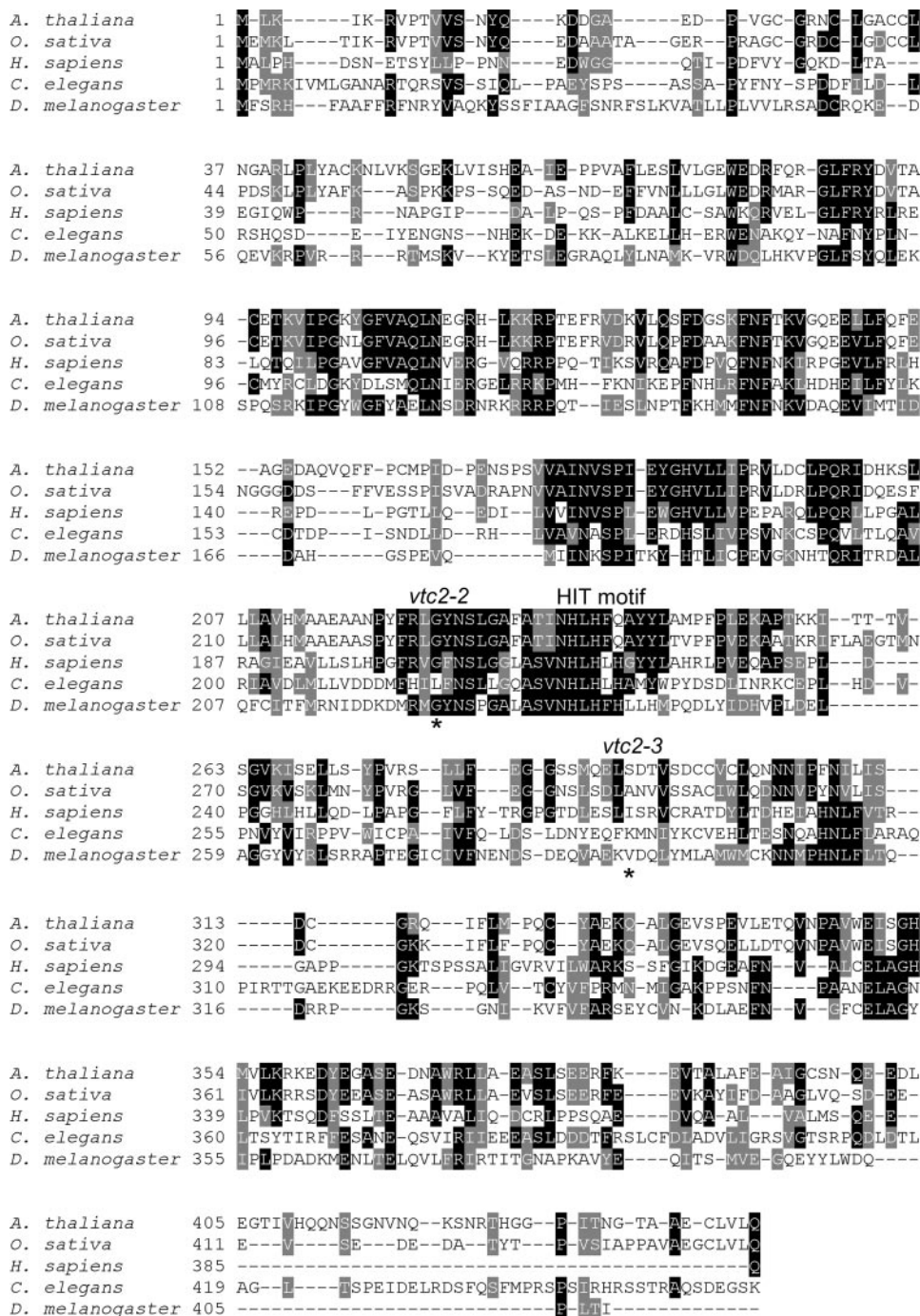
FIGURE 1. **Amino acid alignment of VTC2 homologs.** The *A. thaliana* VTC2 sequence is aligned with those of *Oryza sativa*, *Homo sapiens*, *C. elegans* and *Drosophila melanogaster*. Residues *boxed* in *black* are identical in three or more of the proteins. Residues *boxed* in *gray* are chemically similar. *Asterisks* mark the residues that are altered in the *vtc2-2* and *vtc2-3 A. thaliana* mutant strains, namely G224D and S290F. The HIT motif is indicated. Accession numbers include BT006589 for *A. thaliana*, NP_001066338 for *O. sativa*, BAC85370 for *H. sapiens*, NP_001023638 for *C. elegans*, and NP_648319 for *D. melanogaster*.

GDP-D-Man with GME (46.5 μg/ml) for 5.5 h at 26 °C in 50 mM Tris-HCl, pH 7.5. After heating for 3 min at 98 °C, denatured GME was removed by centrifugation and the supernatant (containing 0.77 mM GDP-D-Man, 0.14 mM GDP-L-Gal, and 0.03 mM GDP-L-Gul) was used for determination of the kinetic constants of VTC2 for GDP-L-Gal.

Cloning, Expression, and Purification of A. thaliana VTC2—The *A. thaliana VTC2* coding sequence was amplified by PCR from a cDNA clone (U17921) provided by the Arabidopsis Biological Resource Center at Ohio State University. This clone contains the coding region of the At4g26850 locus on chromosome 4 (GenBank™ accession number BT006589) and was prepared by the Salk Institute Genomic Analysis Laboratory (22). The forward and reverse primers were 5'-CACCATGTTGAAAAT-CAAAAGAG and 5'-TCACT-GAAGGACAAGGCACTCGG. The PCR product was cloned into the Champion pET100/D-Topo vector (Invitrogen), resulting in a construct adding a 36-amino acid N-terminal extension (MRGSHHHHHHGMA-SMTGGQQMGRDLYDDDDKD-HPFT) to the VTC2 protein. The DNA sequence of the insert was confirmed. The plasmid was transformed into *E. coli* BL21 Star (DE3) cells and grown until the optical density at 600 nm reached 0.5–0.6. Protein overexpression was induced with 0.4 mM isopropyl β-D-1-thio-galactopyranoside. After overnight incubation with shaking at 18 °C, cells were harvested, washed, frozen, resuspended in a pH 7.9 buffer containing 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, and lysed by sonication. Clarified

Sigma. All of the sugar nucleotides and sugar phosphates were in the α-configuration. L-Gal-1-P, in the β-configuration, was purchased from Glycoteam (Hamburg, Germany). All other reagents were of analytical grade. Recombinant *A. thaliana* GDP-D-mannose 3',5'-epimerase (GME) containing an N-terminal His tag was prepared as described (21) from a construct provided by Prof. James H. Naismith (University of St. Andrews, UK). GDP-L-Gal was prepared by incubating 0.94 mM

lysate was loaded onto an Ni^{2+}-charged column (Novagen, San Diego, CA) and eluted using a gradient of the resuspension buffer containing 5 mM to 1 M imidazole. Peak VTC2 protein fractions were pooled and buffer was exchanged to 10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol in a 10 kDa cut-off Amicon Ultra Centrifugal Filter Unit (Millipore, Billerica, MA). Protein concentration was determined using a Lowry assay after precipitation with trichlo-
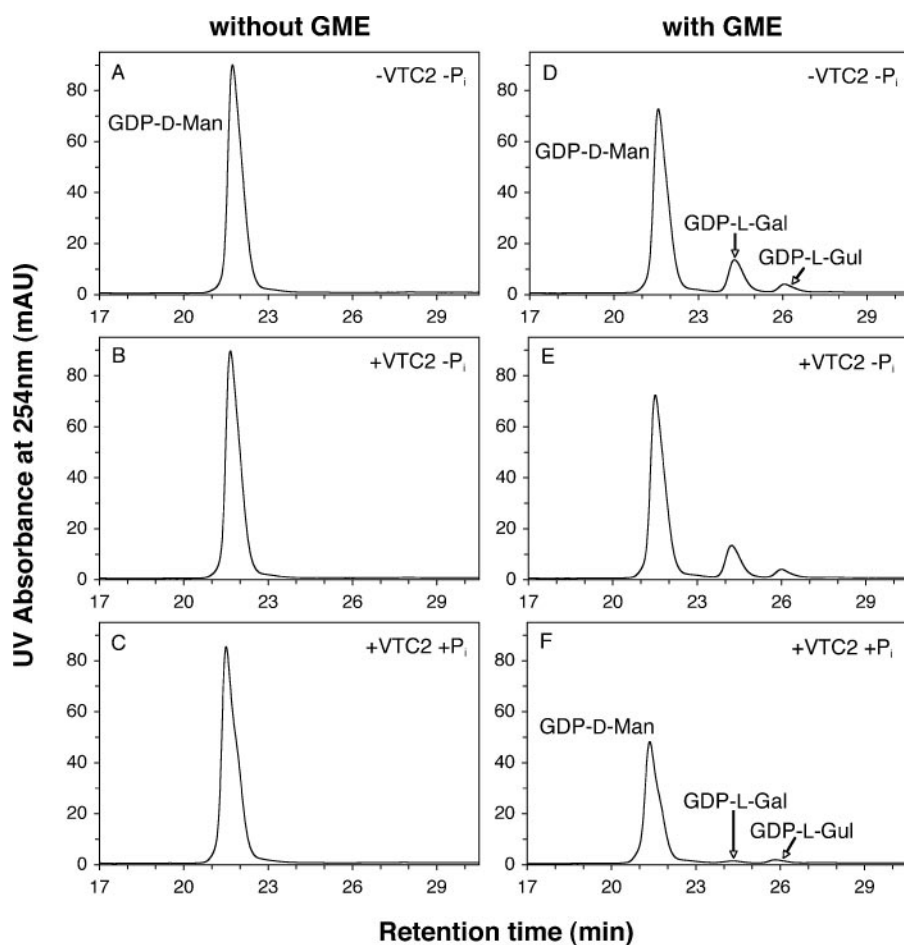
FIGURE 2. **Dependence of VTC2 activity on GME and P$_i$.** GDP-D-Man (*A*) is not a hydrolytic (*B*) nor phosphorolytic (*C*) substrate of VTC2. The equilibrium reaction products of GME activity on GDP-D-Man (*D*), namely GDP-D-Man, GDP-L-Gal, and GDP-L-Gul are not hydrolytic substrates of VTC2 (*E*). However, VTC2 catalyzes a phosphate-dependent reaction that preferentially consumes GDP-L-Gal (*F*). In these experiments, samples were preincubated in the absence of VTC2 for 20 min at 26 °C in the presence of 0.14 mM GDP-D-Man with or without 23 μg/ml GME in a pH 7.5 buffer containing 50 mM Tris-HCl, 2 mM MgCl$_2$, 10 mM NaCl, and 1 mM dithiothreitol. When indicated, sodium phosphate was added at a concentration of 5 mM. Incubations were then continued for 10 min at 26 °C with (*B, C, E, F*) or without (*A, D*) VTC2 at 6.5 μg/ml. The reactions were stopped by heating, and 60 μl of the deproteinized reaction mixtures were adjusted to 100 mM NaCl and analyzed by reverse-phase HPLC as described by Major *et al.* (21) with the exception that an Econosphere C-18 column (5 μm bead size, 4.6 × 250 mm; Alltech Associates, Deerfield, IL) was used. The results shown are representative of at least three independent experiments. Elution times for GMP and GDP were 21 and 43 min, respectively.

roacetic acid. Purified enzyme was stored at −80 °C in 10% glycerol.

Enzymatic Assays—Phosphorylase activity of purified recombinant *A. thaliana* VTC2 was assayed by measuring GDP formation after incubation with various GDP-hexoses in a reaction mixture at pH 7.5 containing 50 mM Tris-HCl, 5 mM sodium phosphate, 2 mM MgCl$_2$, 10 mM NaCl, and 1 mM dithiothreitol. Reactions (26 °C) were initiated with enzyme and stopped after 5 to 60 min by heating at 98 °C for 3 min. After removal of precipitated protein by centrifugation, supernatants were analyzed by anion-exchange HPLC on a Partisil SAX column (10 μm bead size, 4.6 × 250 mm; Alltech Associates, Deerfield, IL) using a Hewlett Packard Series II 1090 liquid chromatograph. A gradient of 0.01– 0.5 M NH$_4$H$_2$PO$_4$, pH 3.7, was used at a flow rate of 2 ml/min. Nucleotides were detected by absorbance at 254 nm using a reference wavelength of 450 nm. GMP, GDP-hexoses, and GDP eluted at ~13, 17, and 24 min,

respectively. To assay the enzymatic activity in the reverse direction (hexose 1-phosphate + GDP → GDP-hexose + P$_i$), GDP-hexose concentration was measured by the anion-exchange HPLC method after incubation of VTC2 with various hexose 1-phosphates and 5 mM GDP as described above, except that sodium phosphate and MgCl$_2$ were omitted. GDP and GDP-hexose concentrations were calculated by comparing the integrated peak areas with those of standard GDP or GDP-D-Man solutions.

Mass Spectrometry—Matrix-assisted laser desorption/ionization mass spectrometry was performed on a Voyager-DE STR MALDI-TOF instrument. GDP-D-Man, GDP-L-Gal and GDP-L-Gul were purified by reverse-phase HPLC (21) using an Econosphere C-18 column (5 μm bead size, 4.6 × 250 mm; Alltech Associates, Deerfield, IL). Fractions (15 s) were collected, dried, and resuspended in a solution of 0.29 M 3-hydroxypicolinic acid with 9 μM GDP as an internal calibrant in acetonitrile/H$_2$O/trifluoroacetic acid (50/50/0.1, v/v/v). The samples were analyzed in positive ion mode and the mass accuracy was ~10–30 ppm.

RESULTS

In our study of aging in the nematode worm *Caenorhabditis elegans*, we became interested in the *C10F3.4* gene that partially overlaps the *pcm-1* gene encoding the L-isoaspartyl protein methyltransferase (23). We examined the deduced amino acid sequence of *C10F3.4*, and as shown in Fig. 1, found apparent orthologs in invertebrates, vertebrates, and plants, including the *VTC2* gene product of *A. thaliana*. By performing PSI-BLAST analyses (24) of the *C10F3.4* and *VTC2* gene products, we detected a low level of similarity with members of the HIT protein superfamily. HIT enzymes consist of nucleoside monophosphate hydrolases related to Hint, Fhit, Aprataxin, and DcpS and nucleoside monophosphate transferases related to GalT, the D-galactose 1-phosphate uridylyltransferase and Apa1, a diadenosine tetraphosphate phosphorylase (reviewed in Ref. 25). In *A. thaliana*, the *vtc2-2* and *vtc2-3* missense mutations (20) map to either side of the HIT motif (Fig. 1). Since these mutants are vitamin C-deficient (18), we considered the possibility that a step in vitamin C biosynthesis is catalyzed by a HIT enzyme.
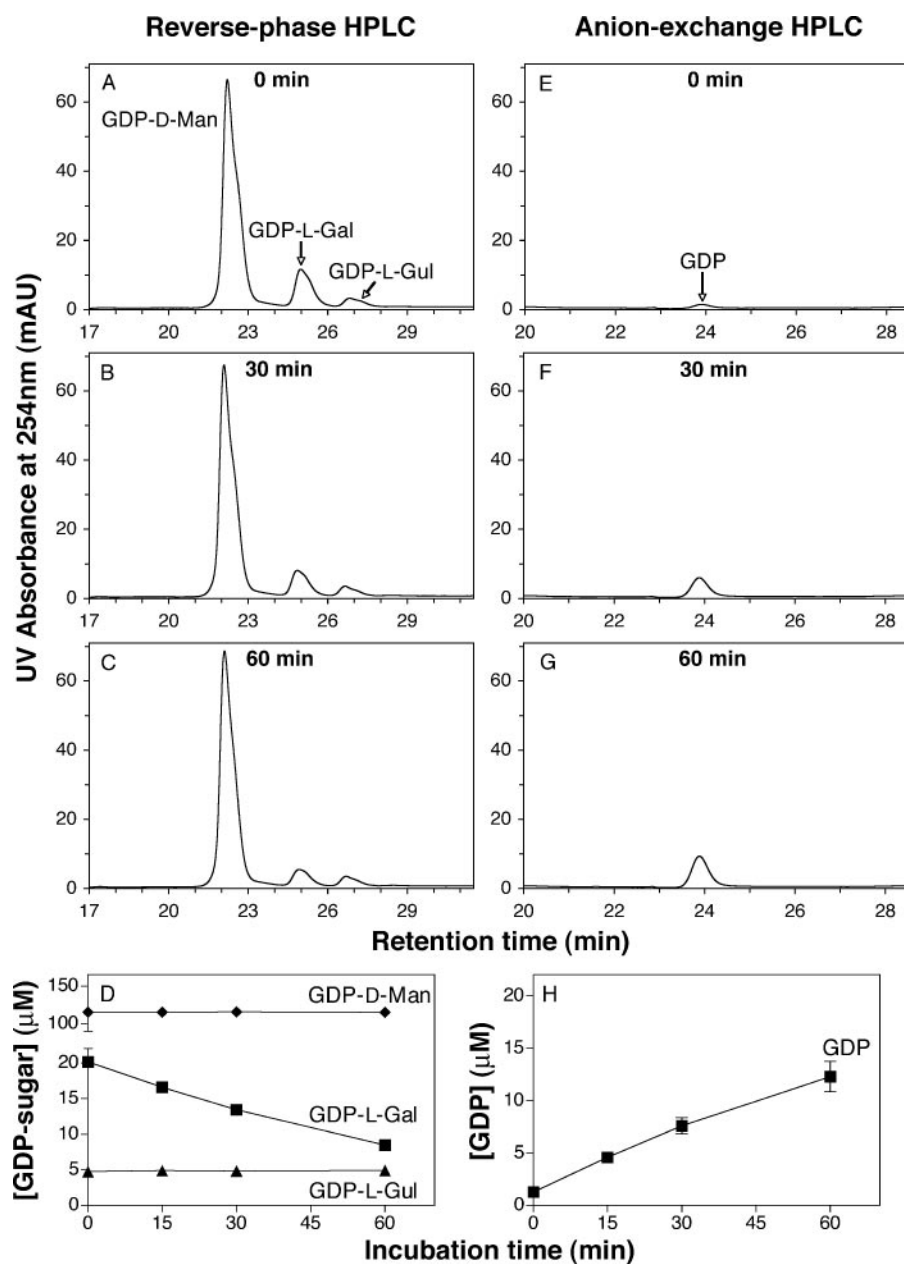
Reverse-phase HPLC **Anion-exchange HPLC**

FIGURE 3. **VTC2-catalyzed phosphorolysis of GDP-L-Gal and formation of GDP.** GDP-D-Man was preincubated for 30 min with GME and 5 mM sodium phosphate as described in Fig. 2. After removal of heat-inactivated (3 min at 98 °C) GME, GDP-hexose consumption and GDP formation were monitored after incubation for the indicated times with VTC2 (6.5 ng/ml). Deproteinized reaction mixtures were analyzed by reverse-phase HPLC (*A–C*) as described in Fig. 2 and anion-exchange HPLC (100 μl samples, *E–G*) as described under "Experimental Procedures." A small amount of GDP (less than 1% of the total GDP-hexose pool) was detected at 0 min as a VTC2-independent hydrolysis product. The HPLC traces shown are representative of three separate experiments. In *D* and *H*, the concentrations of GDP-D-Man, GDP-L-Gal, GDP-L-Gul, and GDP are given as means ± S.D. values calculated from these experiments. Only *error bars* exceeding the symbol sizes are represented.

nucleoside monophosphate moiety of substrates, forming a covalent nucleotidylylated enzyme. In the case of HIT hydrolases, the nucleotidylylated intermediate is labile to water, such that it rapidly decomposes to enzyme plus nucleoside monophosphate. In contrast, the nucleotidylylated intermediate of HIT transferases such as GalT and Apa1 is stable to water and awaits reaction with phosphate (phosphorolysis) or a specific phosphorylated substrate (transfer). Given the lack of conservation of the third His residue in the HIT motif of the plant VTC2 sequence and the requirement of an intact *VTC2* gene for maintenance of the ascorbate pool in *A. thaliana*, we hypothesized that VTC2 may be a GDP-L-galactose phosphorylase that would produce L-Gal-1-P plus GDP in a reaction requiring inorganic phosphate. Despite evidence for the activity, the enzyme that forms L-Gal-1-P remained unknown (11), and it has been widely assumed that such an enzyme would simply hydrolyze GDP-L-Gal into GMP plus L-Gal-1-P (10).

To test the hypothesis that VTC2 catalyzes the phosphorolysis of GDP-L-Gal, we purified recombinant *A. thaliana* VTC2 as an N-terminal His-tagged protein from a bacterial expression system. Because the GDP-L-Gal substrate was not commercially available, we prepared it enzymatically from GDP-D-Man using the recombinant GDP-D-mannose 3′,5′-epimerase (GME) from *A. thaliana* (21) in a coupled assay. We measured the consumption of GDP-D-Man in the presence of VTC2 without and with prior reaction with GME and without and with P$_i$. In the absence of GME, less than 2% of the initial GDP-D-Man was consumed after a 10-min incubation with VTC2, even when P$_i$ was present in the reaction mixture (Fig. 2*C*). GME catalyzes the reversible epimerization of GDP-D-Man to form GDP-L-Gal and, to a lesser extent, GDP-L-gulose (GDP-L-Gul) (21, 26). The substrate and two products can be separated by reverse-phase HPLC (21). We confirmed this chromatographic separation with comparable elution times and an equilibrium product distribution of 82:14:3 for GDP-D-Man, GDP-L-Gal, and GDP-L-Gul (Fig. 2*D*), similar to that reported previously (80:

HIT hydrolases and HIT transferases can be distinguished both by sequence and by enzymatic requirements. HIT hydrolases typically contain an active site motif including His-ϕ-His-ϕ-His, where ϕ is a hydrophobic amino acid. In contrast, the third His of the HIT motif is not conserved in HIT transferases and is frequently a Gln residue (25). As shown in Fig. 1, plant VTC2 sequences have a His-Leu-His-Phe-Gln sequence, whereas animal VTC2 homologs contain a His-Leu-His-(Leu/Phe)-His sequence. All known HIT enzymes use the second His residue of the HIT motif to attack the

15:5; Ref. 21). Furthermore, we confirmed the identification of the GME products by UV absorption spectrum (identical to that of standard GDP-D-Man), as well as by mass spectrometry with the detection of a positive ion of m/z 606, consistent with all three nucleotide sugars.

Addition of VTC2 in the absence of P_i had no effect on the concentration of the GDP-hexoses present in the GME reaction mixture (Fig. 2E). However, in the presence of P_i, VTC2 addition led to a decrease of the three GDP-hexose peaks and, significantly, a modification of the ratio of these peaks (Fig. 2F). After a 10-min incubation with VTC2 and 5 mM P_i, GDP-D-Man, GDP-L-Gal, and GDP-L-Gul were found in the ratio 97:1:2, indicating that VTC2 preferentially acts on GDP-L-Gal. The P_i dependence of this enzymatic reaction is consistent with the prediction from sequence analysis above that VTC2 is a phosphorylase rather than a hydrolase.

To confirm the activity of VTC2 with GDP-L-Gal, we incubated VTC2 in the presence of P_i with the GDP-hexose mixture formed by reaction of GME with GDP-D-Man but under conditions where GME was inactivated by heating prior to the addition of VTC2. Under these conditions, VTC2 only affected the GDP-L-Gal concentration, which decreased linearly with incubation time (Fig. 3, A–D). Parallel analysis of the reaction mixtures by anion-exchange HPLC clearly showed that the consumption of GDP-L-Gal was correlated with the appearance of a peak eluting at ~24 min and identified as GDP by UV spectrum and coelution with standard (Fig. 3, E–H). No formation of GMP was detected in these experiments and the production of GDP was precisely matched to the consumption of GDP-L-Gal.

To characterize the substrate specificity of VTC2, we incubated several nucleoside diphosphate hexoses at a concentration of 1 mM for 10 min (26 °C) in the presence of P_i with or without VTC2 (32.5 μg/ml). NDP-sugar consumption (and NDP formation) were measured by anion-exchange HPLC. No phosphorylase activity was detected when UDP-D-Glc, UDP-D-Gal, or ADP-D-Glc were used as substrates and only a very low activity was found in the presence of GDP-D-Man. However, an almost total conversion of the NDP-sugar to NDP was observed with GDP-D-Glc in the incubation conditions used (data not shown).

Kinetic analysis of the VTC2 phosphorylase activity confirmed that GDP-D-Glc is a good substrate for VTC2 (Table 1). A mixture obtained by GME incubation with GDP-D-Man (and subsequent elimination of GME) was used to determine the kinetic properties of VTC2 for the phosphorolysis of GDP-L-Gal, under conditions where GDP formation reflects only this reaction. VTC2 also exhibited a low K_m value and a high turnover rate with GDP-L-Gal, the values obtained resembling closely those measured with GDP-D-Glc. GDP-D-Man, on the other hand, is a poorer substrate for VTC2 by a factor of ~30,000 in the k_{cat}/K_m term (Table 1).

VTC2 activity could also be measured in the reverse direction by incubating the enzyme in the presence of high concentrations of GDP and various hexose 1-phosphates, and assaying GDP-hexose formation in the absence of added P_i. Table 1 shows that the K_m values of VTC2 for L-Gal-1-P and D-Glc-1-P are more than 3 orders of magnitude higher than for the corre-

TABLE 1

Substrate specificity of the *A. thaliana* VTC2 phosphorylase

K_m and V_{max} values were obtained by fitting the experimental data to the Michaelis-Menten equation using the K_m calculator of the BioMechanic program. Enzymatic turnover numbers were derived from the V_{max} values by using a molecular weight of 53.1 kDa for recombinant His-tagged VTC2 with the assumption that the enzyme preparation is pure. Incubation times and enzyme concentrations were adjusted to obtain initial velocity data. Values are means ± S.D. calculated from at least three individual experiments for each substrate. Enzymatic activities were measured as described under "Experimental Procedures," and substrate concentrations ranged from 3.4–54 μM (GDP-L-Gal), 2.5–100 μM (GDP-D-Glc), 0.25–5 mM (GDP-D-Man), and 2.5–40 mM (hexose 1-phosphates).

	k_{cat}	K_m	k_{cat}/K_m
A. Forward reaction			
	s^{-1}	mM	$s^{-1} M^{-1}$
Substrate			
GDP-L-Gal	64 ± 8	0.010 ± 0.001	6.3 ± 0.9 × 10^6
GDP-D-Glc	23 ± 3	0.0044 ± 0.0016	5.7 ± 2.3 × 10^6
GDP-D-Man	0.093 ± 0.011	0.52 ± 0.15	1.9 ± 0.3 × 10^2
B. Reverse reaction			
Substrate			
L-Gal-1-P	6.0 ± 0.4	45 ± 7	133 ± 12
D-Glc-1-P	12 ± 0.5	29 ± 4	412 ± 50
D-Man-1-P	0.0013 ± 0.0001	54 ± 8	0.025 ± 0.001
D-Gal-1-P	0.023 ± 0.002	67 ± 3	0.34 ± 0.02

sponding GDP-sugar derivatives. The k_{cat} terms for the reverse reaction with these hexose 1-phosphates are, however, relatively high (2–10-fold lower than those found in the direct reaction) and exceed by 2 and 3 orders of magnitude those measured with D-Gal-1-P and D-Man-1-P, respectively. Thus, VTC2 functions much better in the reverse reaction with L-Gal-1-P or D-Glc-1-P than with D-Man-1-P or D-Gal-1-P, confirming the forward reaction products and the specificity.

DISCUSSION

Our demonstration that VTC2 catalyzes the conversion of GDP-L-Gal to L-Gal-1-P completes the identification of genes encoding the enzymes of the Smirnoff-Wheeler pathway of ascorbate synthesis in plants (Fig. 4). We characterize VTC2 as a GDP-L-Gal/GDP-D-Glc phosphorylase. The phosphate requirement of VTC2 and its production of GDP are consistent with the presence of a Gln residue at position 5 of the HIT motif (25). Two well characterized HIT transferases are GalT and Apa1. GalT reacts with UDP-D-Glc to produce a uridylylated enzyme intermediate plus D-Glc-1-P. The uridylylated enzyme is then intercepted by D-Gal-1-P to produce UDP-D-Gal (27). In the case of Apa1, the substrate is diadenosine tetraphosphate, which reacts to form adenylylated enzyme plus ATP. The adenylylated intermediate, stable to water, is phosphorolyzed by P_i to produce an ADP product (28). It can thus be predicted that VTC2, in the presence of GDP-L-Gal or GDP-D-Glc, forms a guanylylated enzyme intermediate at His-238, which is phosphorolyzed by P_i to yield free enzyme and GDP.

As shown in Fig. 4, conversion of D-Glc to L-ascorbic acid through the Smirnoff-Wheeler pathway takes a remarkable 10 steps, 8 of which are required simply to convert D-Glc to L-Gal. L-Gal, representing the epimerization product of D-Gal at four carbon atoms, is rarely found in nature (29), emphasizing the importance of this portion of the pathway. Because two of the interconverted substrates and products of GME, namely GDP-D-Man and GDP-L-Gal, are utilized in biosynthesis of cell wall polysaccharides (30, 31) and/or protein glycosylation (32), the

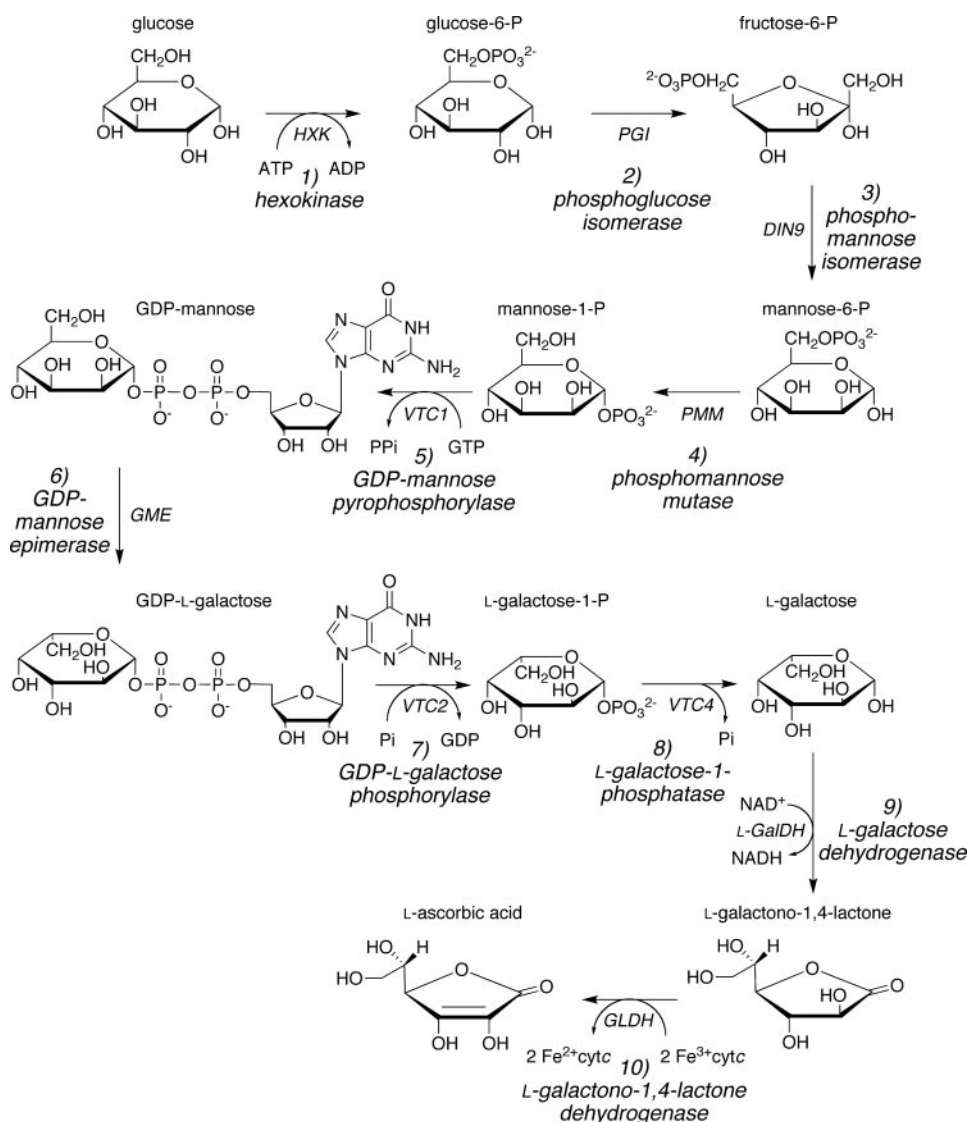# GDP-L-Galactose Phosphorolysis by VTC2 in Ascorbate Synthesis

FIGURE 4. **Smirnoff-Wheeler pathway from D-glucose to L-ascorbic acid.** The forward, on-pathway reactions are depicted, with sugars in the D-configuration unless otherwise designated. The pathway begins with six steps used to produce GDP-Man and GDP-L-Gal for protein glycosylation and/or cell wall biosynthesis. In the seventh and committing step for L-ascorbic acid synthesis, the GDP-L-Gal product of GME is recognized by VTC2, the GDP-L-Gal phosphorylase, to produce L-Gal-1-P. In the eighth step, L-Gal is generated by VTC4, the L-Gal-1-P phosphatase. L-Gal is then oxidized to L-galactono-1,4-lactone and L-ascorbic acid by the NAD$^+$-dependent and ferric cytochrome c-dependent enzymes, L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase.

Wheeler pathway, a commodity sugar is converted to a specialty sugar by virtue of epimerization prior to substrate selection by the HIT enzyme (Fig. 4).

VTC2 exhibits striking substrate specificity for GDP-L-Gal over GDP-D-Man and no apparent activity on GDP-L-Gul. This high selectivity of VTC2 for one of the GME products pulls the equilibrium of the GME-catalyzed reversible reaction in favor of GDP-D-Man conversion to GDP-L-Gal, which is made irreversible in the cell by the activity of the specific L-Gal-1-P phosphatase, VTC4 (14, 15). The specificity of these enzymes suggests that the main flux of ascorbate biosynthesis passes through the L-galactose branch, represented in Fig. 4, and not a putative L-gulose branch through formation of L-gulono-1,4-lactone, the vitamin C precursor in animals. The L-gulose branch has been suggested as a possible alternative ascorbate biosynthesis pathway (26), but enzymes for it have not been identified in plants (9).

While VTC2 exhibits strong discrimination against the GME equilibrium products, GDP-D-Man and GDP-L-Gul, and against ADP-D-Glc, UDP-D-Gal, and UDP-D-Glc, we were surprised at the lack of discrimination between GDP-L-Gal and GDP-D-Glc. In the forward direction, both substrates are converted to hexose 1-phosphates with a specificity constant of $\sim 6 \times 10^6$ s^{-1} M^{-1}. In the reverse direction, while the enzyme is nearly inert at converting D-Man-1-P, the C2

first committed step in the production of ascorbic acid is catalyzed by VTC2. Thus, VTC2 activity may be regulated to coordinate cellular needs for ascorbate with needs for cell wall synthesis and protein glycosylation.

In the Leloir pathway of galactose utilization, cells evolved a pathway to convert D-Gal to D-Glc equivalents by coupling a HIT family transferase (GalT) with an epimerase (UDP-D-galactose 4-epimerase) (27). Similar enzymology is used in the Smirnoff-Wheeler pathway, in which an epimerase (GME) is coupled to a HIT family phosphorylase (VTC2) to convert D-Man to L-Gal. In the former case, a specialty sugar is converted to a commodity sugar. The Leloir pathway moves forward by supplying D-Glc as UDP-D-Glc, using GalT to convert D-Gal-1-P plus UDP-D-Glc to D-Glc-1-P plus UDP-D-Gal, and epimerizing UDP-D-Gal to UDP-D-Glc. In the Smirnoff-

epimer of D-Glc-1-P, or the D-form of Gal-1-P, the enzyme has robust activity on D-Glc-1-P. In fact, the enzyme is three times more efficient at forming GDP-D-Glc than GDP-L-Gal. There are two potential explanations for the lack of specificity. First, although GDP-D-Glc is apparently formed in plants (33) where it can be used for the synthesis of cell wall polysaccharides (34), this metabolite may be spatially separated from VTC2, such that no evolutionary selection against D-Glc-1-P formation has occurred. In this regard it will be important to determine whether plant GDP-Man pyrophosphorylase (VTC1), which is responsible for GDP-D-Man formation in the Smirnoff-Wheeler pathway, discriminates against GDP-D-Glc formation as reported for the mammalian ortholog of VTC1, GDP-Man pyrophosphorylase B (35). Alternatively, if VTC1 does not discriminate against D-Glc-1-P and significant GDP-D-Glc is

formed, the activity of VTC2 on this metabolite could serve to recycle D-Glc-1-P such that there is a second chance to make the fructose and mannose phosphates required for ascorbate synthesis.

Of the four Arabidopsis genes whose mutations result in vitamin C deficiency, the roles of three have now been established. The function of *VTC3* remains unclear. Vitamin C turnover is apparently not affected in the *vtc3* mutant (11) suggesting that *VTC3* is not involved in ascorbate catabolism. Furthermore, the conversion of D-[U-^{14}C]Man to [^{14}C]ascorbic acid has been reported to be decreased in the *vtc3* mutant (11). Thus, *VTC3* may encode a protein involved in the regulation of the Smirnoff-Wheeler pathway or participating in an alternative ascorbate biosynthesis pathway.

Finally, what can we say about the role of the *C10F3.4* gene product of *C. elegans* that initiated our interest in this project? While we detect small amounts of vitamin C in extracts of these worms (data not shown), we do not find orthologs of other distinctive enzymes of the Smirnoff-Wheeler pathway. The presence of a VTC2 homolog in humans, in which a role in vitamin C synthesis is unlikely, suggests that a different function is conserved in animals, potentially in glucose metabolism. Ongoing work is designed to further dissect the function of Arabidopsis VTC2 and to determine the functions of the apparent animal orthologs.

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