A Second GDP-L-galactosePhosphorylase in Arabidopsis en Route to Vitamin C

COVALENT INTERMEDIATE AND SUBSTRATE REQUIREMENTS FOR THE CONSERVED REACTION

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The Arabidopsis thaliana VTC2 gene encodes an enzyme that catalyzes the conversion of GDP-L-galactose to L-galactose 1-phosphate in the first committed step of the Smirnoff-Wheeler pathway to plant vitamin C synthesis. Mutations in VTC2 had previously been found to lead to only partial vitamin C deficiency. Here we show that the Arabidopsis gene At5g55120 encodes an enzyme with high sequence identity to VTC2. Designated VTC5, this enzyme displays substrate specificity and enzymatic properties that are remarkably similar to those of VTC2, suggesting that it may be responsible for residual vitamin C synthesis in vtc2 mutants. The exact nature of the reaction catalyzed by VTC2/VTC5 is controversial because of reports that kiwifruit and Arabidopsis VTC2 utilize hexose 1-phosphates as phosphorolytic acceptor substrates. Using liquid chromatography-mass spectroscopy and a VTC2-H238N mutant, we provide evidence that the reaction proceeds through a covalent guanylylated histidine residue within the histidine triad motif. Moreover, we show that both Arabidopsis VTC2 and VTC5 enzymes catalyze simple phosphorolysis of the guanylylated enzyme, forming GDP and L-galactose 1-phosphate from GDP-L-galactose and phosphate, with low reactivity of hexose 1-phosphates as phosphorolytic acceptors. Indeed, the endogenous activities from Japanese mustard spinach, lemon, and spinach have the same substrate requirements. These results show that Arabidopsis VTC2 and VTC5 proteins and their homologs in other plants are enzymes that guanylate a conserved active site His residue with GDP-L-galactose, forming L-galactose 1-phosphate for vitamin C synthesis, and regenerate the enzyme with phosphate to form GDP.

Vitamin C (L-ascorbic acid) is the most abundant soluble antioxidant in plants, in which it plays crucial roles in protection against oxidative damage and is used as a cofactor for several enzymes. It is synthesized via reactions initially proposed by Wheeler et al. (1) that are distinct from those used in vitamin C biosynthesis in animals. Although the Smirnoff-Wheeler pathway, arising from GDP-mannose and involving L-galactose formation, might not be the only route to vitamin C biosynthesis in plants (alternative pathways arising from myo-inositol and methylgalacturonate or involving L-gulose formation have been proposed; for reviews, see Refs. 2 and 3), it is undoubtedly the pathway that has received the strongest biochemical and genetic support in recent years (4–9).

Of the four loci (VTC1–4) that have been found to be mutated in vitamin C-deficient Arabidopsis thaliana plants (10, 11), three are now known to encode enzymes involved in the Smirnoff-Wheeler pathway. VTC1 encodes GDP-mannose pyrophosphorylase (12), whereas VTC4 encodes L-Gal-1-P 3-phosphatase (8). In 2007 the VTC2 gene product was identified as the enzyme that produces L-Gal-1-P from GDP-L-galactose, which completed the characterization of the 10 enzymatic steps leading from d-glucose to L-ascorbic acid (13, 14). VTC2 is a member of the d-galactose-1-phosphate uridylyltransferase/Apa1 nucleoside monophosphate transferase branch of the histidine triad (HIT) protein superfamily (15). The function of the VTC3 gene remains unknown. Significantly, all vtc mutants identified so far are only partially deficient in vitamin C (11).

In the wake of the identification of VTC2 as the L-Gal-1-P-forming enzyme, three issues remain unresolved. First, are vtc2-2 and vtc2-3 null mutations? If so, is there a VTC2-independent pathway for making vitamin C that might be responsible for the viability of the mutant plants? Second, consistent with the sequence relationship of VTC2 as a HIT protein, does this enzyme proceed through a guanylylated histidine intermediate? Third, does the guanylated intermediate of VTC2 become resolved by simple phosphorolysis (i.e. transfer to phosphate) as we reported (13) or by transfer to a hexose 1-phosphate as reported by others (14)?

Here we find that vtc2-2 and vtc2-3 mutant proteins are nearly devoid of enzymatic activity and that a VTC2 homologous enzyme, termed VTC5 (16), has nearly identical enzymatic characteristics. We find that VTC2 activity and formation of a covalent guanylylated intermediate both depend on

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3 The abbreviations used are: Gal-1-P, galactose 1-phosphate; HIT, histidine triad; HPLC, high performance liquid chromatography.
Phosphorylase Activity of Arabidopsis VTC2 and VTC5

His-238. Finally, we clearly show that there is no requirement for a hexose 1-phosphate substrate for VTC2, VTC5, or for the endogenous 1-Gal-1-P-forming activities from extracts of four different plant species.

EXPERIMENTAL PROCEDURES

Materials—ADP-d-Glc, GDP-d-Glc, GDP-d-Man, UDP-d-Gal, UDP-d-Glc, d-Gal-1-P, d-Glc-1-P, d-Man-1-P (all of these sugars are in the α-configuration), GDP-β-L-Fuc, and GDP were from Sigma. l-Gal-1-P, in the β-configuration, was purchased from Glycotech (Hamburg, Germany). All other reagents were of analytical grade. GDP-β-L-Gal, synthesized and purified as described (17), was provided by Prof. Shinichi Kitamura (Osaka Prefecture University). This preparation was further purified by the reverse-phase HPLC method described in Linster et al. (13). Fractions containing GDP-β-L-Gal were lyophilized, resuspended in H2O, and stored at −20°C.

A. thaliana (ecotype Wasseleskiina) plants used in this study had either been grown on soil or under sterile conditions on agar-solidified Murashige and Skoog medium supplemented with sucrose. Seeds were sown on soil and germinated for 2 days at 4°C before being transferred to the UCLA greenhouse where they were grown at 22°C for 21 days with 16 h of light (100–200 μmol m−2 s−1). Plants were then harvested and stored at −80°C until extracted for protein. For growth on agar-solidified medium, seeds were surface-sterilized (50% (v/v) sodium hypochlorite, 0.1% (w/v) SDS for 10 min with gentle agitation) and then washed 5 times in sterile water. The seeds, resuspended in sterile water, were vernalized for 2 days at 4°C before being sown on Murashige and Skoog medium (Sigma) supplemented with 1% (w/v) sucrose and 0.7% (w/v) agar in Magenta G-7 boxes (Sigma). After 10 days of growth at 22°C under continuous light (~40 μmol m−2 s−1), plants were harvested, flash-frozen in liquid nitrogen, and then stored at −80°C until used for protein extraction. Similar GDP-d-Glc and GDP-β-L-Gal phosphorylase activities were measured in extracts obtained from plants grown under the two conditions described. Japanese mustard spinach (Brassica rapa var. komatsuna), lemon tree (Citrus limon), and maize (Zea mays) leaves were freshly harvested from plants grown in the outside garden of the UCLA greenhouse, whereas tobacco (Nicotiana rustica) leaves were collected from greenhouse grown plants. Kiwifruit leaves were harvested from a female plant purchased at a local nursery. Finally, fresh spinach leaves were purchased at a local supermarket. All the leaves were washed with deionized water and then stored at −80°C until used for protein extraction.

Cloning of A. thaliana VTC2 and VTC5—The A. thaliana VTC2 and VTC5 cDNAs were cloned as described in Linster et al. (13). A similar approach was taken to clone the A. thaliana VTC5 cDNA. In short, the VTC5 coding sequence was PCR-amplified from the U11937 clone containing the coding sequence of the At5g55120 gene (prepared by the Salk Institute Genomic Analysis Laboratory (18) and provided by the Arabidopsis Biological Resource Center at Ohio State University) using the following forward and reverse primers: 5’-CACCATGGTGGTGAAGATCAGAAAGAGATTCC and 5’-TCATTTTCCCCACACAGCCCTCTTTTCTTTTG. The resulting DNA was cloned into the Champion pET100/D-Topo vector (Invitrogen), and this plasmid was transformed into Escherichia coli BL21 Star (DE3) cells. The DNA sequences of both the VTC2 and VTC5 inserts were confirmed.

Preparation of VTC2 Mutant Proteins—VTC2 mutants were constructed by the PCR and DpnI method (19) using Pfu polymerase (Stratagene, La Jolla, CA) and DpnI (New England Biolabs, Ipswich, MA). The pET100/D-Topo plasmid containing the VTC2 coding sequence was used as the mutagenesis template. The sense strand mutagenic primers used for the amino acid substitutions were 5’-CATAGTCAAGCTGATTAACACAGCCCAACAGTTGGG for G224D, 5’-CTATCAATCTCTCAGGCTTATT for H238N, and 5’-CTATGCAAGACATTTGATGTTCCAGA for S290F. The underlined nucleotides indicate the mutations, which were confirmed by DNA sequencing. Plasmids containing the mutant cDNAs were used to transform E. coli BL21 Star (DE3) cells for purification of mutant VTC2 proteins, designated VTC2-G224D, VTC2-H238N, and VTC2-S290F.

Overexpression and Purification of Recombinant VTC2, VTC2 Mutants, and VTC5—Recombinant wild-type and mutant VTC2 enzymes as well as wild-type VTC5 protein were overexpressed and purified as described in Linster et al. (13) with the exception that the lysis buffer contained 0.5 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin. Protein concentration was determined using a Lowry assay after precipitation with trichloroacetic acid. Purified enzyme was stored at −80°C in 10% glycerol.

Activity Assays of Recombinant VTC2, VTC2 Mutants, and VTC5—The phosphorylase activity of recombinant VTC2, VTC2 mutants, and VTC5 was assayed as described in Linster et al. (13). In short, GDP formation was measured by anion-exchange HPLC after incubation of recombinant enzyme with various GDP-hexose substrates in a reaction mixture, pH 7.5, containing 50 mM Tris- HCl, 5 mM MgCl2 (unless otherwise indicated) sodium phosphate, 2 mM MgCl2, 10 mM NaCl, and 1 mM dithiothreitol. Reactions (26°C) were initiated by the addition of enzyme and stopped after the indicated times by heating at 98°C for 3 min. To assay the enzymatic activity in the reverse direction, GDP-hexose formation was measured by the same HPLC method after incubation of the enzyme with the indicated hexose 1-phosphate and 5 mM GDP as described above except that sodium phosphate and MgCl2 were omitted from the reaction mixture.

The GDP-β-L-Gal-hexose 1-phosphate guanylyltransferase activities of recombinant VTC2 and VTC5 were measured by replacing the sodium phosphate used in the phosphorylase assay by the indicated concentrations of d-Glc-1-P or d-Man-1-P and by monitoring GDP-d-Glc or GDP-d-Man formation by the same anion-exchange HPLC method than the one used for the phosphorylase assay.

GDP and GDP-hexose concentrations were calculated by comparing the integrated peak areas with those of standard GDP or GDP-Man solutions. The GDP-β-L-Gal (substrate of the transferase activity) and GDP-d-Glc (product of the transferase activity) peaks partially overlapped. To estimate the total peak area of the GDP-d-Glc (which elutes just before GDP-β-L-Gal) formed, we generally split the peak at the summitt and multiplied the left half-peak area by 2. However, because of an
asymmetry of the peaks toward the right, this can lead to an underestimation of the GDP-(d)-Glc concentrations and, thus, the GDP-(d)-Gal-d-Glc-1-P guanyllytransferase activities by as much as 35%.

**Preparation and Assay of Partially Purified Plant Extracts—**A. thaliana extracts were prepared from whole plants. All other plant extracts used in this study were derived from leaves. Plant tissue was ground to a fine powder under liquid nitrogen and resuspended in 3 volumes of extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin) per gram of extract powder. For extracts prepared from kiwifruit leaves, the extraction buffer was added in a 4:1 (v/w) ratio, and it additionally contained 2% (w/v) polyvinypolypyrrolidone (Sigma). For all plant tissues, the resuspended powder was then centrifuged for 20 min (4 °C) at 20,000 × g, and the resulting supernatant filtered through one layer of Miracloth. This preparation was either stored at −80 °C or immediately subjected to ammonium sulfate fractionation. Protein precipitating between 35 and 50% saturating ammonium sulfate (ICN Biomedicals, Ultra Pure) was resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% glycerol, and 1 μg/ml leupeptin. These fractions were desalted on protein desalting spin columns in 10 mM Tris, pH 7.5 (Pierce) immediately before being used for activity measurements. GDP-(d)-Glc/GDP-(l)-Gal phosphorylase and GDP-(l)-Gal-hexose-1-phosphate guanylyltransferase activities were assayed as described for recombinant VTC2 and VTC5 proteins, except that NaCl and MgCl2 were omitted from the reaction mixtures.

**Phosphorylase Activity of Arabidopsis VTC2 and VTC5**

**TABLE 1**

**Phosphorylase activities of wild-type and mutant VTC2 enzymes**

Activities were measured as described under “Experimental Procedures” in the presence of 5 mM P. Wild-type VTC2, VTC2-H238N, VTC2-S290F, and VTC2-G224D were assayed at final concentrations of 6.8 ng/ml, 23.2 μg/ml, 18.4 μg/ml, and 18.0 μg/ml, and reactions were stopped 10 or 30 min after the addition of wild-type or mutant VTC2 enzymes, respectively. Values are given as the means ± S.D. calculated from at least three independent measurements.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphorylase activity</th>
<th>25 μM GDP-(l)-Gal</th>
<th>50 μM GDP-(d)-Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTC2 (wild type)</td>
<td>15,100 ± 2,100</td>
<td>14,200 ± 2,100</td>
<td></td>
</tr>
<tr>
<td>VTC2-H238N</td>
<td>0.51 ± 0.07</td>
<td>0.27 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>VTC2-S290F</td>
<td>0.50 ± 0.12</td>
<td>0.36 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>VTC2-G224D</td>
<td>0.054 ± 0.006</td>
<td>0.013 ± 0.12</td>
<td></td>
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</tbody>
</table>

**TABLE 2**

**Comparison of the substrate specificities of A. thaliana VTC2 and VTC5**

K_m and V_max values were obtained by fitting the experimental data to the Michaelis-Menten equation using the Km calculator of the BioMechanic.org program. Enzymatic turnover numbers were derived from the V_max values by using a molecular mass of 53.1 and 52.4 kDa for recombinant His-tagged VTC2 and VTC5, respectively, with the assumption that the enzyme preparations were pure. Incubation times and enzyme concentrations were adjusted to obtain initial velocity data. Values are the means ± S.D. calculated from 2–4 individual experiments for each substrate. Enzymatic activities were measured as described under “Experimental Procedures,” and substrate concentrations ranged from 2.5 to 50 μM (GDP-(l)-Gal), 2.5 to 100 μM (GDP-(d)-Glc), 0.25 to 5 μM (GDP-(d)-Man), 0.1 to 5 μM (Pi), and 2.5 to 40 μM (hexose-1-phosphates).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (μM)</th>
<th>V_max (μmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP-(l)-Gal</td>
<td>0.0079</td>
<td>0.0083 ± 0.0032</td>
</tr>
<tr>
<td>GDP-(d)-Glc</td>
<td>0.0044</td>
<td>0.012 ± 0.002</td>
</tr>
<tr>
<td>GDP-(d)-Man</td>
<td>0.52</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Pi</td>
<td>0.76</td>
<td>0.22 ± 0.04</td>
</tr>
</tbody>
</table>

**Reverse reaction**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (μM)</th>
<th>V_max (μmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP-(l)-Gal</td>
<td>45 ± 7</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>GDP-(d)-Glc</td>
<td>29 ± 4</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

**Forward reaction**

<table>
<thead>
<tr>
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<tr>
<td>GDP-(l)-Gal</td>
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<td>0.0044</td>
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</tr>
<tr>
<td>GDP-(d)-Man</td>
<td>0.52</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.4</td>
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</table>

a For these substrates the values given for the VTC2 enzyme were taken from Linster et al. (13).

b Measured in the presence of GDP-(d)-Glc 25 μM.

c Measured in the presence of GDP-(d)-Glc 50 μM.
80% solvent A (0.1% trifluoroacetic acid in water) and 20% solvent B (0.1% trifluoroacetic acid in acetonitrile). After a 5-min wash at 20% B, proteins were eluted using a gradient changing from 20% to 60% B in 40 min, with a final increase to 100% B during an additional 5 min, all at a flow rate of 100 μl/min. An API III+ mass spectrometer (PE Sciex) was tuned and calibrated as described previously (20) to yield a mass accuracy of 0.02%.

RESULTS

VTC2 Point Mutants Lack Enzymatic Activity—Three mutations in the VTC2 gene (vtc2-1, vtc2-2, and vtc2-3) are known to lead to partial vitamin C deficiency in A. thaliana plants (11). Whereas the vtc2-1 mutation consists in a G to A base change at the predicted 3’ splice site of intron 5, the vtc2-2 and vtc2-3 mutations have been found to correspond to mis-sense mutations leading to G224D and S290F substitutions, respectively (21). To test the effect of the latter mutations on enzyme activity, we have introduced them in the VTC2 coding sequence by site-directed mutagenesis. We have also prepared a mutant allele in which the predicted catalytic residue of VTC2, His-238 (see below), has been replaced by an Asn residue.

Overexpression and purification yielded relatively high amounts of the recombinant VTC2-H238N protein (Fig. 1). Production of the VTC2-G224D and VTC2-S290F mutants proved more difficult, and only low amounts of protein could be detected (Fig. 1). SDS-PAGE analysis of the pellets and supernatants obtained after centrifugation of the bacterial lysates showed that the VTC2-S290F mutant was mostly insoluble and that the VTC2-G224D mutant appeared to have been entirely degraded (data not shown).

With the VTC2-H238N and VTC2-S290F preparations, we detected only very low residual phosphorylase activities both in the presence of GDP-L-Gal and GDP-D-Glc (Table 1). These residual activi-
ties were, however, significantly higher than those detected in the presence of the VTC2-G224D preparation and, thus, do not seem to be contributed by bacterial contaminants. The phosphorylase activities of the VTC2-H238N mutant were at least 30,000-fold lower than the wild-type activities. Because of the low level of soluble expression of the VTC2-S290F mutant, we were unable to calculate its activity relative to the wild-type enzyme.

**VTC5 Is a VTC2-paralogous Enzyme**—The vtc2-2 and vtc2-3 mutants had previously been shown to contain ~30 and 50%, respectively, of the ascorbate present in wild-type plants (11). The very low activities detected with the VTC2-G224D and VTC2-S290F mutants, thus, raised the question of the origin of these residual vitamin C contents. BLAST searches revealed that the *A. thaliana* genome encodes a protein sharing high sequence identity with VTC2. We cloned and overexpressed the corresponding gene (At5g55120) and purified the expression product (Fig. 1, lane 2) using the same procedures as those used to produce His-tagged VTC2 (13). We found that At5g55120 encodes a GDP-1-Gal phosphorylase whose biochemical properties closely resemble those of VTC2 (Table 2) and which might, thus, account for the residual vitamin C levels found in vtc2 mutants. During the course of these studies Dowdle et al. (16) also overexpressed At5g55120 and characterized the gene product, which they named VTC5, as a GDP-1-Gal phosphorylase.

We first characterized the substrate specificity of VTC5 for nucleotide sugars. As shown in Table 2, both VTC2 and VTC5 use GDP-1-Gal and GDP-1-Glc with high catalytic efficiencies, although the values obtained for VTC5 are ~2- and 7-fold, respectively, lower than those calculated for VTC2. GDP-1-Man is a very poor substrate for both enzymes. We could, however, detect significant phosphorylase activities with both enzymes in the presence of GDP-1-Fuc, which is the 6-deoxy derivative of GDP-1-Gal. About 10-fold lower phosphorylase activities were measured in the presence of ~80 μM GDP-1-Fuc (2.4 ± 0.1 μmol min⁻¹ mg of protein⁻¹ for VTC2 and 1.1 ± 0.1 μmol min⁻¹ mg of protein⁻¹ for VTC5) than in the presence of a near-saturating (~50 μM) concentration of the physiological substrate GDP-1-Gal (25 ± 5 μmol min⁻¹ mg of protein⁻¹ for VTC2 and 12 ± 1 μmol min⁻¹ mg of protein⁻¹ for VTC5). Similar to the situation with VTC2 (13), no phosphorylase activity could be detected with VTC5 when UDP-1-Glc, UDP-1-Gal, or ADP-1-Glc was used as a substrate (all tested at 0.5 mM; data not shown). Using a mixture obtained by incubation of GDP-1-Man with GDP-1-mannose 3',5'-epimerase and containing GDP-1-Man, GDP-1-Gal, and GDP-1-gulose in a 82:15:3 ratio, we could not detect any GDP-1-gulose phosphorylase in the presence of VTC5 and under conditions where about 80% consumption of GDP-1-Gal was observed (data not shown); a similar result was found previously with VTC2 (13).

We then examined the affinity for phosphate as a substrate. VTC5 showed a 2.5- and 3.5-fold higher affinity for P<sub>I</sub> than VTC2 in the presence of GDP-1-Gal and GDP-1-Glc, respectively.

Finally, we compared the ability of VTC5 and VTC2 to carry out the reverse reaction (hexose-1-P + GDP → GDP-hexose + P<sub>I</sub>). Both enzymes are much more efficient in phosphorylizing GDP-1-Gal or GDP-1-Glc than in catalyzing the corresponding reverse reactions (Table 2). However, this difference in efficiency for the forward and reverse reactions is more pronounced in the case of VTC2. The specificity constants for the forward reactions with GDP-1-Gal and GDP-1-Glc are ~26,000- and 14,000-fold, respectively, higher than the ones for the reverse reactions in the case of VTC2, whereas for VTC5 these ratios amount to ~6400- and 1500-fold, respectively. Taken together, these results suggest that VTC2 and VTC5 catalyze similar reactions with similar kinetics.

**VTC2 Forms a His-238-dependent Guanylylated Intermediate**—The alignment of plant VTC2 sequences and their vertebrate and invertebrate homologs revealed the presence of a conserved motif (His-ϕ·His-ϕ·His/Gln, where ϕ is a hydrophobic amino acid) characteristic of the members of the HIT protein superfamily (13, 14). HIT enzymes consist of nucleoside monophosphate hydrolases and nucleoside monophosphate transferases which attack the α-phosphate of the monophosphonucleoside moiety of their substrates by the second His of the HIT motif, forming a covalent nucleotidylated intermediate (15). While the nucleotidylated intermediate is simply hydrolyzed in the case of HIT hydrolases, this intermediate is stable to water in HIT transferases and awaits reaction with P<sub>I</sub> (phosphorylisis) or a specific phosphorylated substrate (transfer). We know that *A. thaliana* VTC2 requires the presence of P<sub>I</sub> to convert its GDP-hexose substrates (GDP-1-Gal and GDP-1-Glc) to GDP and the corresponding hexose 1-phosphates (1-Gal-1-P and 1-Glc-1-P, respectively) (13). However, given the relatively low sequence similarity with other members of the HIT protein superfamily, it was important to experimentally confirm the predicted catalytic mechanism. We, therefore, prepared a point mutant in which the second His of the HIT motif of Arabidopsis VTC2 (His-238) is replaced by an Asn residue. We have shown above that this substitution reduces the catalytic activity by at least 30,000-fold (Table 1).

We then incubated wild-type VTC2 and VTC2-H238N without P<sub>I</sub> in the presence or absence of GDP-1-Glc and analyzed the four resulting preparations by liquid chromatography-mass spectroscopy. As shown in Fig. 2, a decrease of the mass peak corresponding to the non-modified enzyme and the appearance of a new peak of 344 higher mass, were observed after reaction with GDP-1-Glc when wild-type VTC2, but not when VTC2-H238N, was used in the incubation. Because a 345-Da mass increase is expected for covalently bound GMP, each sample was compared to its respective control (no GMP). As shown in Fig. 2, the mass peaks corresponding to the non-modified enzyme and the expected mass peaks for the modified forms of the two enzymes were detected in the samples with GDP-1-Glc and not in the control samples. These results confirm the formation of the His-238-GDP-1-Glc intermediate by VTC2 and VTC2-H238N in vitro.
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These observations suggest formation of an enzyme intermediate guanylated on the His-238 residue and strongly support the catalytic mechanism expected for a member of the d-galactose-1-phosphate uridylyltransferase/Apa1 nucleoside monophosphate transferase branch of the HIT protein superfamily (15).

A. thaliana VTC2 and VTC5 Are Both Highly Specific for P₄ as the Guanylyl Acceptor—In our previous study on VTC2 (13), the specificity of the enzyme for the guanylyl acceptor had not been investigated. To test whether VTC2 could catalyze the conversion of its GDP-hexose substrate in the presence of guanylyl acceptors other than P₄, we incubated the enzyme (at two different concentrations) with GDP-L-Gal and in the absence or presence of P₄, P₄, d-Man-1-P, and d-Man-1-P. As shown in Fig. 3G, only P₄ gave rise to a significant GDP-L-Gal consumption when recombinant A. thaliana VTC2 was used at a concentration of 0.026 μg/ml. At a 10-fold higher enzyme concentration, total conversion of GDP-L-Gal to GDP was measured in the presence of P₄ (Fig. 3B), whereas only a very low conversion (~4%) of GDP-L-Gal to GDP-d-Glc could be detected in the presence of d-Man-1-P (Fig. 3E). Even at these high enzyme concentrations, no formation of GDP-d-Man or GTP (two compounds readily detectable with the HPLC method used; data not shown) could be measured in the presence of d-Man-1-P (Fig. 3D) or P₄ (Fig. 3C), respectively. Very similar results were obtained when VTC5 was used instead of VTC2 in these experiments (data not shown).

We then measured the effect of P₄ and d-Glc-1-P concentration on the GDP-L-Gal phosphorylase and GDP-L-Gal-p-Glc-1-P guanylyltransferase activities, respectively, of VTC2 (Fig. 4) and VTC5 (Fig. 5). At all acceptor concentrations tested, the VTC2 and VTC5 activities were considerably lower in the presence of d-Glc-1-P than in the presence of P₄. More particularly, at concentrations of 5 mM P₄ or d-Glc-1-P (which are close to physiological concentrations reported for P₄ in plants (22, 23) but are at least 100-fold higher than those measured for d-Glc-1-P (24)), VTC2 activity was ~33-fold higher in the presence of P₄ than in the presence of d-Glc-1-P. For reasons that are yet unclear, the addition of P₄ or d-Glc-1-P concentrations higher than 5 or 20 mM, respectively, leads to unexpectedly high enzyme activities when recombinant VTC2 or VTC5 enzymes are used (data not shown). However, in the concentration range used in Figs. 4 and 5, Michaelis-Menten kinetics were observed, and kinetic parameters could be estimated. For VTC2, Kₘ values of 1.8 and 26 mM and Vₘax values of 19 and 2.6 μmol min⁻¹ mg⁻¹ of protein⁻¹ were calculated in the presence of P₄ and d-Glc-1-P, respectively, indicating that this enzyme is more than 100-

![FIGURE 3. Recombinant VTC2 is highly specific for P₄ as the guanylyl acceptor used during conversion of GDP-L-Gal to L-Gal-1-P. VTC2 (0.026 or 0.26 μg/ml) was incubated for 30 min in the presence of 25 μM GDP-L-Gal and various potential guanylyl acceptors as described under “Experimental Procedures.” The deproteinized samples were analyzed by anion-exchange HPLC as described in Linster et al. (13) to monitor GDP-L-Gal consumption and the possible production of GDP, GTP, GDP-d-Man, and GDP-p-Glc. The traces obtained after incubation with high VTC2 concentration (0.26 μg/ml) are shown (panels A–E). They are representative of three separate experiments. Panel F shows the separation of authentic GMP, GDP-p-Glc, GDP-L-Gal, and GDP by the HPLC method used. GDP-d-Man co-elutes with GDP-p-Glc, and GTP elutes at ~40 min (not shown). In panel G, the concentrations of GDP-L-Gal measured after incubation in the described conditions are given as means ± S.D. values calculated from three separate experiments. mAU, milliabsorbance units.]
fold more efficient as a GDP-\(\text{L-Gal}\) phosphorylase than as a GDP-\(\text{L-Gal}\)-\(\text{d-Glc-1-P}\) guanylyltransferase. In the case of VTC5, we found \(K_m\) values of 0.96 and 6.8 mM and \(V_{\text{max}}\) values of 15 and 2.4 \(\mu\text{mol min}^{-1}\text{mg of protein}^{-1}\) in the presence of \(\text{P}_i\) and \(\text{d-Glc-1-P}\), respectively, indicating that this enzyme is about 45-fold more efficient as a phosphorylase than as a transferase. No GDP-\(\text{L-Gal}\)-\(\text{d-Man-1-P}\) guanylyltransferase activity could be detected with either VTC2 or VTC5 and up to 40 mM \(\text{d-Man-1-P}\) (Figs. 4 and 5).

Confirmation of \(\text{P}_i\) as the Preferred Guanyl Acceptor for GDP-\(\text{L-Gal}\) to \(\text{L-Gal-1-P}\) Conversion Measured in Plant Tissue Extracts—We next wanted to test whether our observations concerning the acceptor specificity of recombinant VTC2 and VTC5 could be confirmed with partially purified plant extracts as the enzyme source. Using ammonium sulfate fractions of \(A.\ thaliana\) whole plant extracts, we measured \(\text{P}_i\)-dependent GDP formation which correlated with \(\text{P}_i\)-dependent GDP-\(\text{d-Glc}\) or GDP-\(\text{L-Gal}\) consumption, reflecting the sum of the VTC2 and VTC5 activities of these preparations (Table 3). We also found that GDP-\(\text{d-Glc}\) and GDP-\(\text{L-Gal}\) phosphorylase activities could be detected in ammonium sulfate precipitates of extracts of Japanese mustard spinach, lemon, spinach, and maize leaves but not of kiwifruit or tobacco leaves (Table 3). Except for the maize leaf extract, slightly higher phosphorylase activities were found when GDP-\(\text{L-Gal}\) (25 \(\mu\text{M}\)) was used instead of GDP-\(\text{d-Glc}\) (50 \(\mu\text{M}\)).

However, we found that the levels of phosphorylase activity ranged widely among different plants (Table 3). The highest activities were measured in ammonium sulfate fractions of Japanese mustard spinach leaves, lemon leaves, and \(A.\ thaliana\) whole plants; much lower activities were found in similar extracts of spinach and maize leaves.

In these same plant extracts we also measured transferase activity as \(\text{d-Glc-1-P}\) and \(\text{d-Man-1-P}\)-dependent GDP-\(\text{d-Glc}\) and GDP-\(\text{d-Man}\) formation, respectively, in the presence of GDP-\(\text{L-Gal}\) (Table 3). The guanylyltransferase activities found in the various plant extracts in the presence of GDP-\(\text{L-Gal}\) were generally at least 10-fold lower than the GDP-\(\text{L-Gal}\) phosphorylase activities (except for the maize leaf extract, where the transferase activity was only about 2-fold lower than the phosphorylase activity). No guanylyltransferase activity could be detected in the presence of \(\text{d-Man-1-P}\) and GDP-\(\text{d-Gal}\), except for the lemon leaf extract, where this activity was, however, more than 100-fold lower than the corresponding GDP-\(\text{L-Gal}\) phosphorylase activity. Finally, in tobacco or kiwifruit leaf
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**TABLE 3**

Phosphorylase and transferase activities in various partially purified plant extracts

Activities were assayed as described under “Experimental Procedures.” GDP-β-Glc and GDP-β-Man were added at concentrations of 25 and 50 μmol/l respectively. Protein concentrations and incubation times ranged from 0.04 to 1.6 mg/ml and 15 to 120 min, respectively, according to the plant extract used. Plant extracts, prepared as ammonium sulfate fractions as described under “Experimental Procedures,” were incubated in the absence or presence of 5 mM guanyl acceptor (P~i, d-Glc-1-P, or d-Man-1-P) to measure P~i-dependent GDP (phosphorylase activity) or hexose 1-phosphate-dependent GDP-d-Glc or GDP-d-Man (transferase activities) formations, which were then used to calculate specific activities. Data are the means ± S.D. values calculated from three separate measurements.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Activity (GMP donor/GMP acceptor)</th>
<th>GDP-β-Glc/P~i</th>
<th>GDP-β-Gal/P~i</th>
<th>GDP-β-Glc/d-Glc-1-P</th>
<th>GDP-β-Gal/d-Man-1-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td></td>
<td>424 ± 225</td>
<td>698 ± 194</td>
<td>74 ± 49</td>
<td>−1.8 ± 2.8</td>
</tr>
<tr>
<td>Lemon</td>
<td></td>
<td>1312 ± 329</td>
<td>3626 ± 789</td>
<td>351 ± 45</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>Spinach</td>
<td></td>
<td>45 ± 8</td>
<td>88 ± 19</td>
<td>7.1 ± 2.8</td>
<td>−0.45 ± 0.74</td>
</tr>
<tr>
<td>Maize</td>
<td></td>
<td>52 ± 9</td>
<td>37 ± 9</td>
<td>16 ± 10</td>
<td>3.5 ± 2.9</td>
</tr>
<tr>
<td>Kiwifruit</td>
<td></td>
<td>6.6 ± 2.0</td>
<td>12 ± 16</td>
<td>−17 ± 25</td>
<td>−4.0 ± 1.7</td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
<td>−8.4 ± 4.7</td>
<td>−18 ± 5.1</td>
<td>3.8 ± 0.8</td>
<td>0.26 ± 1.0</td>
</tr>
</tbody>
</table>

*Except for Arabidopsis, all plant extracts were prepared from leaf tissue. Whole plants were used for Arabidopsis protein extraction.*

**FIGURE 6.** Effect of P~i, d-Glc-1-P, and d-Man-1-P concentrations on the GDP-β-Gal phosphorylase or GDP-β-Gal-hexose 1-phosphate transferase activities in partially purified extracts of A. thaliana (panel A) and Japanese mustard spinache leaves (panel B). The phosphorylase and transferase activities shown were assayed as described under “Experimental Procedures” in the presence of 25 μmol/L GDP-β-Gal and the indicated concentrations of P~i and d-Glc-1-P or d-Man-1-P, respectively. Incubation times and protein concentrations were adjusted to obtain initial velocity data. The curves shown represent ideal saturation curves obtained by fitting the activities measured in the presence of P~i and d-Glc-1-P to the Michaelis-Menten equation. The results shown are means ± S.D. values calculated from at least three (panel A) or two (panel B) separate measurements.

Extracts, transferase activities were close to or below the detection limit (Table 3).

Two of the partially purified plant extracts were used to estimate and compare the kinetic constants of their GDP-β-Gal phosphorylase and GDP-β-Gal-d-Glc-1-P guanylyltransferase activities (Fig. 6). In the Japanese mustard leaf extracts, K~m~ values of 1.4 (±0.2, n = 2) and 13 (±1, n = 3) μmol and V~max~ values of 4.8 (±0.1, n = 2) and 0.99 (±0.11, n = 3) nmol min~−1~ mg of protein~−1~ were estimated in the presence of P~i~ and d-Glc-1-P, respectively. Similar K~m~ values were found for P~i~ and d-Glc-1-P in Arabidopsis extracts (1.1 ± 0.3 and 18 ± 2 μmol/L, n = 4, respectively), whereas the corresponding V~max~ values (1.3 ± 0.1 and 0.28 ± 0.03 nmol min~−1~ mg of protein~−1~, n = 4, respectively) were ~4 times lower in these extracts than in the Japanese mustard spinach extracts. It can, thus, be calculated that the catalytic efficiencies (V~max~/K~m~) of the GDP-β-Gal phosphorylase activity of these extracts are about 45-fold (Japanese mustard spinach leaf extract) and 75-fold (A. thaliana extract) higher than the catalytic efficiencies of the GDP-β-Gal-d-Glc-1-P guanylyltransferase activities in these same extracts. No GDP-β-Gal-d-Man-1-P guanylyltransferase activity could be detected in either of these extracts in the presence of d-Man-1-P concentrations of up to 40 μmol (Fig. 6). These results show that in both recombinant enzyme and in tissue extracts, the predominantly catalyzed reaction is phosphorolysis.

**DISCUSSION**

Of the three mutations in the Arabidopsis VTC2 gene (vtc2-1, vtc2-2, and vtc2-3) known to lead to vitamin C deficiency (11), two have been identified as mis-sense mutations leading to G224D (vtc2-2) and S290F (vtc2-3) substitutions (21). At the time these mutations were discovered the VTC2 function was still unknown. With the recent identification of VTC2 as a GDP-β-Gal phosphorolase (13), it became possible to test the effect of the mis-sense mutations on this activity to increase our understanding of the enzymology of VTC2 and to confirm the physiological role of VTC2 as catalyzing a reaction involved in plant vitamin C biosynthesis. The S290F substitution led to an enzymatic activity of the purified preparation that was ~30,000-fold lower than that of the wild-type VTC2 preparation. However, the purity of the mutant protein being much lower, we could not compare the specific activities of the wild-type VTC2 and VTC2-S290F enzymes. We were unable to stably express the VTC2-G224D protein; from our results it is, thus, unclear whether this enzyme is stable in cells or indeed has any activity. During the course of this work, Dowdle et al. (16) also characterized the VTC2-S290F and VTC2-G224D mutants. The authors found no activity with the latter mutant, although the issue of its stability was not addressed. They were, however, able to show that the VTC2-S290F mutant displayed a
10-fold higher $K_m$ for $P_i$ and a 5-fold lower $k_{cat}$ than the wild-type enzyme, resulting in a 50-fold lower catalytic efficiency for the mutant enzyme. Taken together, these results indicate that the relatively high residual vitamin C levels measured in vtc2-2 (~30% that of wild-type content) and vtc2-3 (~50% of wild-type content) mutants (11, 16) are unlikely to be accounted for by the residual VTC2 activities. Although the existence of alternative pathways for vitamin C synthesis has been proposed in plants, the finding that the A. thaliana genome contains a gene (At5g55120) sharing high similarity with the VTC2 gene indicated the existence of an enzyme able to maintain flux through the Smirnoff-Wheeler pathway in the absence of VTC2.

Accordingly, we found that the product of the At5g55120 gene is also highly efficient as a GDP-L-Gal/GDP-d-Glc phosphorylase. This partially confirms the results obtained during the course of our studies by Dowdle et al. (16), who designated this enzyme VTC5. However, the kinetic properties of the GDP-L-Gal phosphorylase activities of recombinant VTC2 and VTC5 published by Dowdle et al. (16) differ quite substantially from the ones found in this study. Compared with our results, Dowdle et al. (16) found higher $K_m$ values for GDP-L-Gal (250 versus 7.9 μM for VTC2 and 667 versus 8.3 μM for VTC5), lower $k_{cat}$ values (2.0 versus 27 s⁻¹ for VTC2 and 2.7 versus 13 s⁻¹ for VTC5), and lower $K_m$ values for $P_i$ (0.25 versus 2.4 mM for VTC2 and 0.13 versus 1.0 mM for VTC5). The use of different expression plasmids and/or different enzyme activity assays (direct HPLC assay in this study and coupled assay in Dowdle et al. (16)) might account for the discrepancy between the results obtained. The fact that the $K_m$ values for GDP-L-Gal and $P_i$ obtained with our recombinant VTC2 and VTC5 preparations are closer to the values obtained with native pea enzyme ($K_m$ values of 18 μM and 1.1 mM for GDP-L-Gal and $P_i$, respectively (16)) as well as with partially purified A. thaliana extracts ($K_m$ value of 1.1 mM for $P_i$; this study) suggests that the values found in this study may reflect more closely the properties of the native enzymes.

Comparison of the kinetic properties of VTC2 and VTC5 did not reveal any fundamental differences between these two enzymes. As for VTC2 (Ref. 13 and this study), we found that VTC5 displays a strong substrate preference for GDP-L-Gal and GDP-d-Glc over GDP-L-Fuc and GDP-d-Man, and that UDP-d-Glc, UDP-d-Gal, and ADP-d-Glc are not substrates at all. Furthermore, as for VTC2, VTC5 does not seem to phosphorolyz GDP-L-gulose, a compound formed, in addition to GDP-L-Gal, from GDP-d-Man by GDP-d-mannose 3’,5’-epimerase (25) in the reaction catalyzed upstream of GDP-L-Gal phosphorolysis in the Smirnoff-Wheeler pathway. Neither VTC2 nor VTC5 does, thus, seem to participate in the putative vitamin C synthesis pathway involving L-gulose and L-gulono-1,4-lactone formation, one of the alternative pathways that has been proposed for vitamin C synthesis in plants (25). Both VTC2 and VTC5 catalyze the phosphorolysis of GDP-L-Gal and GDP-d-Glc much more efficiently than the corresponding reverse reactions.

The reason for the conservation of two proteins with the same biochemical function in Arabidopsis is not clear. VTC2 and VTC5 might act in different subcellular compartments, but this has not yet been investigated. Dowdle et al. (16) showed that VTC2 and VTC5 are both expressed in Arabidopsis leaf, stem, root, flower, and silique tissue but that the mRNA expression level of VTC5 is 100–1000-fold lower than the one of VTC2 in all these tissues. Exposure of Arabidopsis plants to high light led to increased ascorbate contents as well as increased expression of VTC5 and, more importantly, VTC2 (16). Jasmonate treatment also led to ascorbate accumulation in Arabidopsis as well as induction of both the VTC2 and VTC5 genes (26). The expression of the VTC5 gene seemed, however, to be more responsive to jasmonate treatment as well as to induction by ozone exposure (26), which might indicate that VTC5 only contributes significantly to ascorbate synthesis under certain stress conditions. Importantly, with the identification of VTC5, it has now become possible to find out whether pathways other than the Smirnoff-Wheeler pathway significantly contribute to plant vitamin C synthesis. During the course of this study, Dowdle et al. (16) found that double mutants in VTC2 and VTC5 are unable to grow unless supplemented with ascorbate, demonstrating not only that the Smirnoff-Wheeler pathway is the only physiologically significant source of vitamin C, at least in A. thaliana, but also that ascorbate is required for seedling viability.

Members of the d-galactose-1-phosphate uridylyltransferase/Apa1 branch of the HIT protein superfamily transfer the monophosphonucleoside moiety of their substrate either to $P_i$ or to a specific phosphorylated compound. d-galactose-1-phosphate uridylyltransferase, for example, transfers UMP from UDP-d-Glc to d-Gal-1-P forming d-Glc-1-P and UDP-d-Gal (27). Using a recombinant VTC2 homolog from kiwifruit, Laing et al. (14) detected GDP-L-Gal transferase activities in the presence of $P_i$, $P_i$, and a series of hexose 1-phosphates including d-Man-1-P and d-Glc-1-P. The highest activities were measured in the presence of the hexose 1-phosphates, and they proposed d-Man-1-P as the most likely in vivo guanylyl acceptor. They reported that recombinant A. thaliana VTC2 also showed transferase activity with similar properties to the kiwifruit enzyme (14). These results contrast with the characterization of VTC2 as a GDP-L-Gal phosphorylase by our group (13) and by Dowdle et al. (16). Additionally, in this study we could not detect any formation of GDP-d-Man or GTP from GDP-L-Gal in the presence of VTC2 or VTC5 and d-Man-1-P or $P_i$, respectively. We measured a small transferase activity with d-Glc-1-P, but this activity was 100- to 45-fold less than the phosphorolysylase activity of VTC2 and VTC5, respectively.

In plants, cytosolic concentrations have been estimated at 3–7 mM for $P_i$ (22, 23) and at ~50 μM for d-Glc-1-P (24). Given the $K_m$ values of 0.96 to 1.8 mM found here for $P_i$ and 6.8 to 26 mM for d-Glc-1-P, it seems clear that the phosphorolysylase reaction will be responsible for almost all of the VTC2 and VTC5 activities in plant cells. The striking preference of the VTC2 and VTC5 phosphorolysylase activities for GDP-d-Glc over GDP-d-Man as the GDP-hexose donor indicates that these enzymes have binding sites that can accommodate d-Glc-1-P, but not, or much less efficiently, d-Man-1-P, which would explain the lack of GDP-L-Gal-d-Man-1-P guanylyltransferase activity that we observe.

It is difficult to rationalize the differences between the studies supporting a transferase (14) rather than a phosphorolysylase activity.
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(13, 16) activity for VTC2/VTC5 (28). We find no evidence for differences in the acceptor specificity of the enzymatic reaction in different plant species. GDP-\(\alpha\)-Gal phosphorylase activities could be readily detected in partially purified extracts prepared from Arabidopsis thaliana plants as well as Japanese mustard spinach and lemon leaves. These extracts also displayed some GDP-\(\alpha\)-Gal-\(\alpha\)-Glc-1-P guanylylttransferase activity, which was, however, ~10-fold lower than the corresponding phosphorylase activities. It is possible that the different assay methods may have contributed to the divergent results. In this study, GDP and GDP-hexoses formed by the VTC2 and VTC5 phosphorylase and transferase activities, respectively, were detected directly by HPLC. Laing et al. (14) employed an indirect enzymatic assay to measure release of the \(\alpha\)-Gal-1-P product (formed in both the phosphorylase and transferase reactions). This enzymatic assay was based on the use of two E. coli-expressed coupling enzymes (\(\alpha\)-Gal-1-P phosphatase and \(\alpha\)-Gal dehydrogenase) and the spectrophotometric measurement of NADH formation. Although these enzymes have been claimed to be specific for their substrates (6, 7, 14), low activities found with \(\alpha\)-Gal-1-P phosphatase and some other hexose 1-phosphates (\(\alpha\)-myo-inositol 1-phosphate, \(\beta\)-Gal-1-P, 4-Man-1-P, 4-Glc-1-P (7)) as well as a possible inhibition of \(\alpha\)-Gal-1-P phosphatase by P\(\alpha\), may complicate the coupled assay.

Based on the GDP-\(\alpha\)-Gal-\(\alpha\)-Man-1-P guanylyltransferase activity they measure, Laing et al. (14) proposed a VTC2 cycle in which the biosynthesis of \(\alpha\)-Gal-1-P from \(\alpha\)-Man-1-P can be sustained by the action of only two enzymes: VTC2 and GDP-\(\beta\)-mannose 3',5'-epimerase. In a subsequent review, Wolucka and Van Montagu (28) extended this initial proposal by including a putative GDP-\(\beta\)-mannose 2'-epimerase and by taking into account the double specificity of VTC2 for GDP-\(\alpha\)-Gal and GDP-\(\alpha\)-Glc, leading to a cycle that links photosynthesis with the biosynthesis of vitamin C and the cell-wall metabolism. Considering the very low transferase activity of VTC2 and VTC5 that we measured in this study, it is unclear whether a VTC2 cycle actually operates in plants.

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REFERENCES


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