The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content

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The gene for one postulated enzyme that converts GDP-L-galactose to L-galactose-1-phosphate is unknown in the L-galactose pathway of ascorbic acid biosynthesis and a possible candidate identified through map-based cloning is the uncharacterized gene At4g26850. We identified a putative function for At4g26850 using PSI-Blast and motif searching to show it was a member of the histidine triad superfamily, which includes D-galactose uridyltransferase. We cloned and expressed this *Arabidopsis* gene and the homologous gene from *Actinidia chinensis* in *Escherichia coli* and assayed the expressed protein for activities related to converting GDP-L-galactose to L-galactose-1-P. The expressed protein is best described as a GDP-L-galactose-hexose-1-phosphate guanyltransferase (EC 2.7.7.), catalyzing the transfer of GMP from GDP-L-galactose to a hexose-1-P, most likely D-mannose-1-phosphate *in vivo*. Transient expression of this *A. chinensis* gene in tobacco leaves resulted in a >3-fold increase in leaf ascorbate as well as a 50-fold increase in GDP-L-galactose-D-mannose-1-phosphate guanyltransferase activity.

GDP-L-galactose pyrophosphorylase | gold kiwifruit | VTC2

Humans and all animals depend on ascorbate (vitamin C) as an essential antioxidant. Humans and several other mammalian species are unable to synthesize ascorbate because of an inactivation of the last gene in the pathway, and so we are dependent on dietary vitamin C, mainly from vegetables and fruits. A significant route for ascorbate biosynthesis in plants is through the L-galactose pathway (1–5), although other pathways may also be operating (6, 7). To date, all of the genes encoding enzymes, and their associated enzymatic activities, for the L-galactose pathway have been identified and at least partially characterized, except for one, an enzyme proposed to convert GDP-L-galactose to L-galactose-1-phosphate. The characterized genes and enzyme activities include the GDP-D-mannose pyrophosphorylase (1, 8, 9), the GDP-D-mannose 3′,5′-Epimerase (10–12), the L-galactose-1-P phosphatase (13, 14), L-galactose dehydrogenase (5, 15, 16), and L-galactono-1,4-lactone dehydrogenase (17, 18). The missing enzyme, which has not been reported as being assayed either as an extracted or purified enzyme activity or as an expressed gene, catalyzes the second committed step to ascorbic acid biosynthesis.

The *VTC2* mutant of *Arabidopsis thaliana* was first identified in a screen for mutants with increased sensitivity to ozone and was characterized as showing especially low ascorbic acid levels (19). The mutated gene was cloned by using a map-based approach (20) and identified as a gene (At4g26850) encoding a novel protein. However, the genes reported to show most similarity to At4g26850 were the similarly uncharacterized *Arabidopsis* gene At5g55120 and other uncharacterized genes from nonplant species. The authors were therefore unable to identify a specific role for Atg26850 in a regulatory or biosynthetic pathway leading to reduced vitamin C levels in the VTC2 mutants (20).

We investigated the function of At4g26850 and a homologous gene from kiwifruit using both BLAST searching and Motif identification as well as gene cloning and protein expression in *Escherichia coli* and enzyme assay of the expressed protein and analysis of its expression patterns in plants. We conclude that the *Arabidopsis* and kiwifruit genes are orthologous, encoding the missing enzyme in the L-galactose pathway, and biochemically function as L-galactose-1-phosphate-hexose-1-phosphate guanyltransferases, transferring GMP from GDP-L-galactose to a hexose-1-phosphate, probably L-mannose-1-P *in vivo*. Expression of the gene using a transient expression system in tobacco leaves resulted in a >3-fold increase in leaf ascorbate compared with that in control leaves as well as the appearance of 50-fold increased rates of transferase activity.

Results

Through BLASTp searches for genes encoding proteins similar to the predicted protein sequence of the uncharacterized *Arabidopsis* gene At4g26850, we initially detected only other plant genes that were also annotated as similar to the VTC2 gene. However, further into the list of matched genes were members of the Interpro HIT family (IPR001310) of proteins that are characterized as nucleotide-binding proteins and hydrolases. The family includes diadenosine tetraphosphate (Ap4A) hydrolase and GalT (D-galactose-1-phosphate-uridyltransferase, class I, EC 2.7.7.12) (21). For example, a rat gene belonging to this GalT family showed an expected value of 1E-37, with 30% identity and 48% similarity over 364 residues of At4g26850. These HIT proteins are usually characterized by the motif HXHXH (where X is a hydrophobic amino acid), although the GalT subgroup (also interpro IPR001937) has the related motif HXHXQ. GalT has been shown to be a member of the HIT family of proteins based on structural analysis (22). We refined this search using PSI-BLAST (23, 24), and a major category of aligned sequences was composed of members of the HIT family with similar descriptions.

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The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database [accession no. EF379384 (EST 319998)].

Abbreviations: GalT, D-galactose-1-phosphate-uridyltransferase; LCMS, liquid chromatography MS; SRM, selective reaction monitoring; SIM, selected ion monitoring; FW, Fresh Weight.

See Commentary on page 9109.

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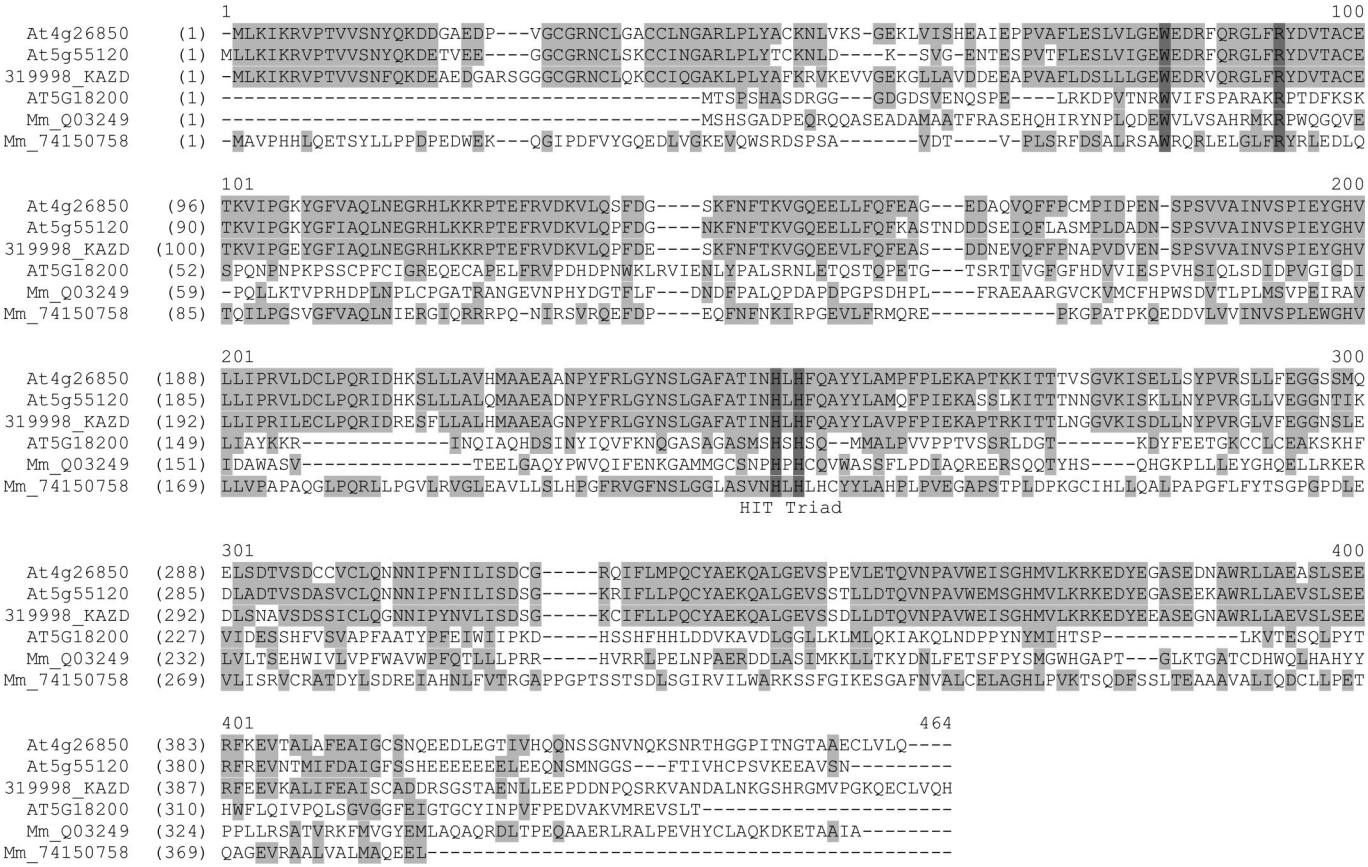

Fig. 1. An alignment of the *A. thaliana* sequence VTC2 with the kiwifruit sequence 319998 and a second *A. thaliana* sequence At5 g55120. Also shown are the *Arabidopsis* enzyme At5 g18200 [coding for a putative UDP-D-glucose-hexose-1-phosphate uridylyltransferase (EC 2.7.7.12)] and the unnamed mouse protein Mm_74150758 (the GenBank accession no.). Identical aligned residues in all five sequences are shown in dark gray, similar residues in light gray. The sequences were aligned by using Clustal X (41) with some manual adjustment. The HIT triad sequence is identified at approximately amino acid residue 250.

Blast searches using At4g26850 of our *Actinidia* EST database revealed 114 ESTs with homology to AT4g26850 of >132,000 ESTs (expect value <1E-10). These came from a range of tissues including petals, fruit, buds and meristems, and leaves. We selected EST 319998 from an *Actinidia chinensis* young fruit library. The two *Arabidopsis* proteins and the kiwifruit protein showed 71–75% identity to each other (Fig. 1).

We then used a selected group of interpro IPR001310 members of the HIT group, plus At4g26850, At5g55120, and EST 319998 [see supporting information (SI) Table 3] and searched for motifs using the MEME web site http://meme.sdsc.edu (25). We identified six significant motifs that were present in all five plant sequences. Five of these motifs were present in four animal sequences, and the remaining animal sequence had four motifs (see SI Table 3). This shows that these proteins are clearly related and belong to the HIT superfamily.

Motif 1 included the diagnostic pattern HxHxH/Q (Fig. 1). Interestingly, the GalT subfamily of the HIT family also shares this HxHxQ pattern, although we were unable to discover other longer motifs in common with this sequence.

From these bioinformatics analyses, and the bioinformatics connection to GalT through the HIT family, it appeared possible that the gene responsible for the ascorbic acid mutant *VTC2* (At4g26850) and its kiwifruit homologue encoded a guanyltransferase. We expressed these genes in *Escherichia coli* in the pET30 vector and purified the protein using the His tag and a Ni chelating column. The protein appeared on an SDS gel at ≈55 kDa and constituted ≈90% of the protein isolated. Controls containing the empty pET30 vector were also treated in the same manner.

We used two assays to characterize the enzyme, using two sources of the substrate GDP-L-galactose. The first assay used the *E. coli* expressed coupling enzymes L-galactose-1-phosphate phosphatase and L-galactose dehydrogenase. The phosphatase is highly specific to L-galactose-1-phosphate, otherwise only significantly dephosphorylating myo-inositol-1-P (14). The dehydrogenase is specific to L-galactose, not reacting with D-mannose or D-galactose or a range of other sugars (15, 16) except for L-gulose. With this latter substrate, L-galactose dehydrogenase showed ≈2.5-fold higher maximum velocity and 30 times the K_m (substrate) resulting in ≈8% activity with L-gulose compared with L-galactose at limiting substrate concentrations. Consequently, our coupled assay would measure primarily L-galactose and also L-gulose. We measured the product formed either by adding the coupling enzymes in the assay and measuring the time course of NADH formed or by stopping the reaction after 10 min by boiling for 3 min and centrifuging. To this latter fixed-time assay, we then either added coupling enzymes to measure L-galactose production or used liquid chromatography MS (LCMS) to measure the products. LCMS was used only to confirm the results of the coupled enzyme reaction and to measure the reverse reaction.

Using both LCMS and the coupled reaction to measure products, it was clear that *E. coli*-expressed kiwifruit EST 319998 and At4g26850 could catalyze the conversion of GDP-L-galactose to L-galactose-1-P. Depending on enzyme concentration, time courses were linear for up to ≈10 min, and the rate of

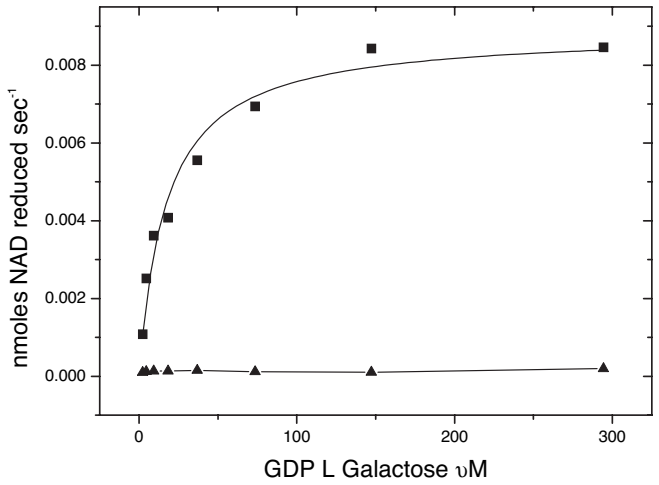

Fig. 2. Response of the GDP-mannose-1-P guanyl transferase to GDP-L-galactose. GDP-L-galactose was made from GDP-D-mannose by using the epimerase as described was in *Methods*, and the concentration that was GDP-L-galactose in the mixture was determined by HPLC. Assays were conducted by using the continuous coupled assay by using 0.029 μg of enzyme per assay. Mannose-1-P concentration was 0.93 and 1.87 mM $MgCl_2$. Other conditions were as described in the text. Squares represent the reaction minus the background run without mannose-1-P. Triangles represent the background values by using HisTrap purified *E. coli* extract (0.006 μg) expressing an empty PET30a vector.

reaction was linear with added enzyme over the range assayed (data not shown). No reaction occurred in the presence of boiled enzyme or empty vector (Fig. 2). D-Mannose-1-P was a significantly better acceptor for the guanyl moiety than phosphate or pyrophosphate, but some reaction was seen with these latter two compounds at physiological concentrations of these substrates (Fig. 3). No NAD reduction activity was seen with GDP-D-mannose without epimerase or with either substrate and without the coupling enzymes (data not shown). Reactions using commercially purchased GDP-L-galactose-1-phosphate had high

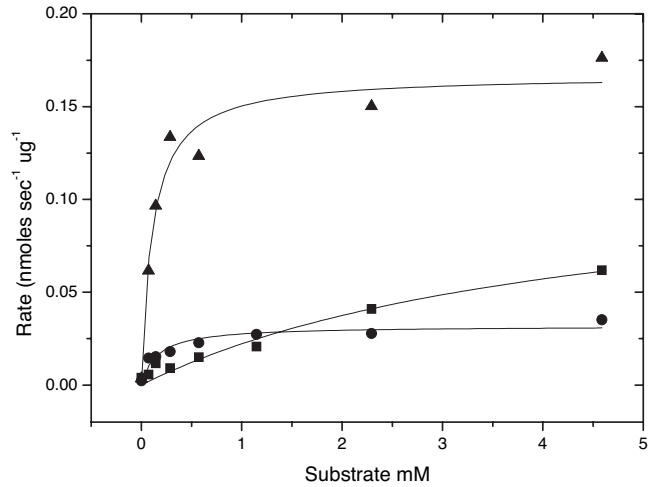

Fig. 3. Response of the enzyme to potential guanyl acceptors. Assays were carried out by using the continuous coupled assay with varying concentrations of inorganic phosphate (square), inorganic pyrophosphate (circle), or D-mannose-1-P (triangle) as the guanyl acceptor. The V_{max} values were 0.12 ± 0.03, 0.032 ± 0.002, and 0.17 ± 0.009 nmol $sec^{-1}\cdot\mu g^{-1}$ protein for the substrates phosphate, pyrophosphate, and D-mannose-1-P, respectively. K_m values were 4.4 ± 2, 0.16 ± 0.05, and 0.11 ± 0.03 mM, respectively. Assays were carried out three times with similar results.

Table 1. Effect of different sugar phosphates acting as guanyl acceptors for the transferase activity

Substrate	Rate, nmol $sec^{-1}\cdot\mu g^{-1}$ protein	SEM	Percent of D-mannose-1-P
α-D-Glucose-1-P	0.35	0.036	106
D-Glucose-6-P	0.08	0.002	24
β-D-Glucose-1-P	0.24	0.05	74
L-Myoinositol-1-P	0.42	0.07	126
D-Galactose-1-P	0.38	0.01	113
D-Mannose-1-P	0.33	0.07	100

Enzyme was assayed by using the epimerase-generated substrate and the continuous coupled assay with other conditions, as described in *Methods*. $n = 6$.

backgrounds because of the contaminating L-galactose-1-phosphate and were assayed by using the fixed-time procedure. This substrate showed a slightly higher rate than that seen with the epimerase-generated substrate. Other guanyl acceptors were tested, and the enzyme was found to accept a wide range of hexose-1-P substrates, although D-glucose-6-P reacted at only ≈25% the rate of the best acceptors (Table 1). The reaction did not require Mg (data not shown), although Mg was included in the coupled assay, because the phosphatase required Mg.

Coupled assays using the expressed *VTC2* gene from *Arabidopsis* (At4g26850) also showed transferase activity (data not shown) with similar properties to the EST 319998.

The products of the reaction were confirmed by using LCMS to be L-galactose-1-phosphate (Table 2). This involved separation of the products of the reaction using LC, which resolved L-galactose from D-mannose and GDP-L-galactose from GDP-D-mannose and confirmation of the identity of the products by their measured mass. Little to no back reaction was detected.

When tobacco leaves were transiently transformed with an *Agrobacterium* clone containing EST 319998 in the vector pGreen mixed with P19 as a suppressor of silencing, activity was detected in the extract of the leaves (Fig. 4). Approximately 2% of the activity found in the 319998 transformed tobacco was seen in the leaves transformed only with P19 (Fig. 4). The low enzyme level in the controls is typical of other enzymes in the L-galactose pathway of ascorbate biosynthesis (W.A.L., unpublished observations).

The activity was present in a range of leaves of different ages injected with *Agrobacterium*. The same leaves transformed with

Table 2. Measurement of transferase activity by LCMS

Substrate	Acceptor	Protein, μg	Protein, nmol $sec^{-1}\cdot\mu g^{-1}$ Coupled assay	LCMS
GDPMan/epim	Mannose-1-P	0.057	0.012	0.0094
GDPMan/epim	None	1.14	0.00038	0.00031
GDPMan/epim	None	0.057	0.00012	0
GDPGal	Mannose-1-P	0.057	0.017	High BG
GDPMan/epim	Ppi	1.14	0.00095	0.0013
GDPMan/epim	Ppi	0.057	0.0026	0.0031
GDPMan	Gal1P	1.14	nm	0
GDPMan	Gal1P	0.057	nm	0
GTP	Gal1P	1.14	nm	0
GTP	Gal1P	0.057	nm	0

Activity was measured by using a fixed time assay at either high- or low-protein concentration and different acceptor and substrate combinations, as shown. Assays were killed by boiling, and aliquots were measured either by using coupling enzymes or by LCMS. GDPMan/epim refers to the substrate generated by the epimerase. nm, not measured.

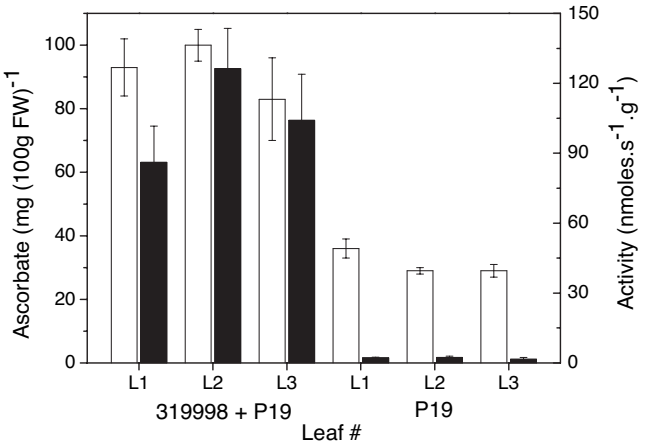

Fig. 4. Effect of transiently expressed 319998 on ascorbate content and enzyme activity in tobacco leaves. See *Methods* for details. White bars represent ascorbate concentration (expressed on a fresh-weight basis) in the leaf, and black bars represent the GDP-L-galactose D-mannose-1-phosphate guanyltransferase activity (expressed on a gram of protein basis). L1, L2, and L3 represent the three youngest leaves that were injected. Error bars are the standard errors of the mean (n = 3–6).

319998 showed a highly significant 3-fold increase in ascorbate compared with the control leaves (Fig. 4). Gel filtration chromatography on G75 and G200 Superdex equilibrated with extraction buffer (without EDTA) of the tobacco extract containing the expressed kiwifruit protein showed the enzyme behaved as a monomer, running at a lower M_r than BSA (67,000) (data not shown).

Discussion

In this paper, we have characterized an *E. coli*-expressed kiwifruit gene homologous to the *A. thaliana* gene At4g26850 as a GDP-L-galactose-D mannose-1-phosphate guanyltransferase. This *Arabidopsis* gene is responsible (20) for the low-ascorbate VTC2 mutant (19). This represents the last remaining unidentified enzyme step in the L-galactose pathway of ascorbate biosynthesis. We have also shown that overexpression of this gene transiently in tobacco results in a 3-fold increase in ascorbate, showing that the gene is likely to be rate-limiting for ascorbate production.

An in-depth bioinformatics analysis of the VTC2 gene indicted that it was a member of the HIT family of genes. This HIT superfamily is named after the consensus motif sequence HxHxH (where x is a hydrophobic amino acid). The three branches of the Histidine Triad superfamily of nucleotide hydrolases and transferases are represented by D-galactose-1-phosphate uridyl transferase [GalT branch; D-galactose-1-phosphate uridyltransferase (EC 2.7.7.12)], the adenosine 5′-monophosphoramide hydrolases (Hint branch), and the diadenosine polyphosphate hydrolase (Fhit branch) (21). GalT transfers UMP from UDP-D-glucose to D-galactose-1-phosphate and is a member of the Leloir pathway (26) where D-galactose is converted to the metabolically useful glucose. Although this GalT enzyme showed little homology or other similarity to the *VTC2* gene, it did share a key motif with *VTC2*, HxHxQ.

The enzyme identified in this study appeared to be rather nonspecific as to the acceptor for the GMP transfer, being able to use a range of hexose-1-P sugars as well as or slightly better than D-mannose-1-P. However, the enzyme did appear not to be effective at transferring the GMP from GDP-L-galactose-1-phosphate to either pyrophosphate or phosphate, suggesting the enzyme is not a pyrophosphorylase or a phosphorylase. We have not tested whether the enzyme was similarly nonspecific for the GDP-hexose donor at this stage, having tested only GDP-L-galactose and GDP-D-mannose (no reaction detected). The turnover number of the enzyme was $\approx 20\ s^{-1}$ within the range of that reported for a number of species in BRENDA (www.brenda.uni-koeln.de for enzyme), EC 2.7.7.12.

Using a transient transformation system in tobacco leaves, we tripled the levels of ascorbate in leaves through overexpression of the transferase gene from kiwifruit. Overexpression of enzymes in the L-galactose pathway has not otherwise resulted in increased ascorbate in leaves (2), although an increase was observed with the last enzyme in this pathway in cell culture (27). Other approaches of overexpressing enzymes in other proposed

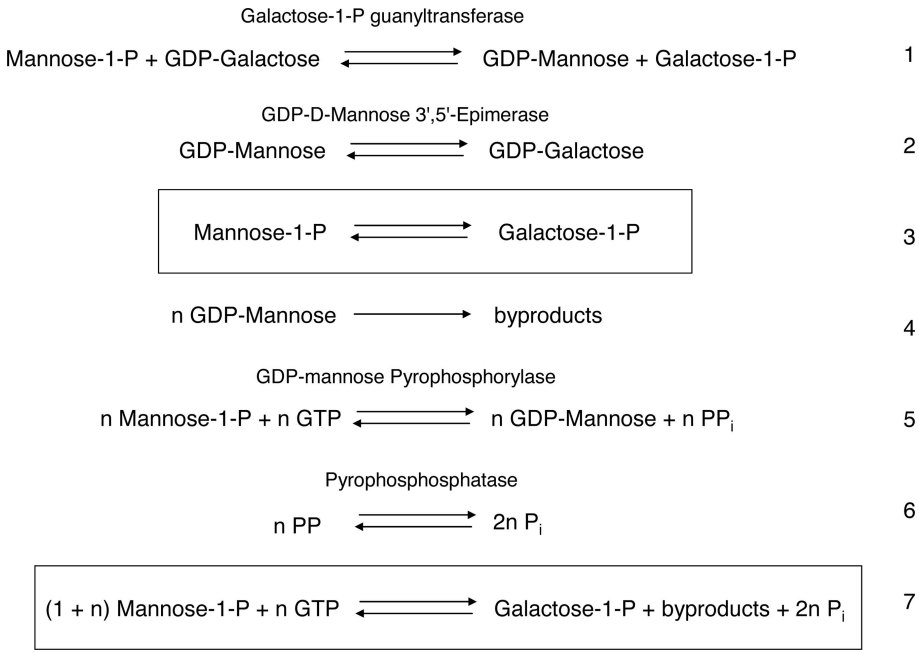

Fig. 5. Reactions converting D-mannose-1-phosphate to L-galactose-1-phosphate. Reactions in boxes represent summations of the reactions above them.

pathways of ascorbate have been reported to increase ascorbate in plant tissue (6, 7, 28) as has overexpression of dehydroascorbate reductase (29).

The biosynthesis of L-galactose-1-phosphate can be accomplished from D-mannose-1-phosphate through the activity of just two enzymes: the new enzyme L-galactose-1-phosphate guanyltransferase and GDP-D-mannose 3′,5′-epimerase (Fig. 5, reaction 3). This conversion presupposes that all of the GDP-D-mannose will be converted into GDP-L-galactose for use by the guanyltransferase in making L-galactose-1-phosphate, with no diversion to other products. It also assumes there is a priming GDP-L-galactose (or GDP-D-mannose) available to start the reaction. However, GDP-D-mannose is also used, for example, in mannan biosynthesis (30) and as a precursor in cell wall biosynthesis (31), which would significantly upset the stoichiometry of the conversion. For example, mucilage in kiwifruit (*Actinidia deliciosa*) leaves contributes ≈2.5 g D-mannose per 100 g fresh weight (FW) (32), whereas cell walls in kiwifruit fruit have 60–100 mg D-mannose per 100 g FW (33) compared with ascorbate at ≈80 mg/100 g FW in green kiwifruit (34). Consequently, the enzyme GDP-D-mannose pyrophosphorylase (*VTC1*) is needed to ensure pools of GDP-D-mannose are maintained and net L-galactose-1-phosphate (and thus ascorbate) and cell wall biosynthesis can both occur (Fig. 5, reaction 7). This suggests n in Fig. 5 will be ≈1 for fruit and 25 for leaves, ignoring turnover of ascorbic acid. The hydrolysis of pyrophosphate will provide the drive to force the reaction in the direction of ascorbate biosynthesis.

Methods

Similarity and Motif Searches. PSI Blast (23, 24) was run for six or more iterations and identified genes further examined for their annotations. Motif searching was done by using MEME (25) with a set of genes as input selected (VTC2, and HIT members including GalT).

Expression of Genes in *E. coli*. The EST 319998 from young fruit of *Actinidia chinensis* and At4g26850 were each cloned into pET30A (Novagene, Palmerston North, New Zealand), their sequence checked and expressed in *E. coli*. The N-terminal His_6 tag was used to purify the protein. An empty vector control was expressed and purified in parallel. Techniques were essentially as described (14). In much of this work, the His protein was further purified on a 5-ml HiTrap Q FF column (GE Healthcare, Auckland, New Zealand), and identical results were obtained with both preparations.

Coupling Enzymes. L-Galactose dehydrogenase [GenBank accession no. AAO18639 (EST 56121), 1.5 μg per assay] was cloned from an EST derived from an *A. deliciosa* (kiwifruit) shoot bud library with a maltose-binding protein presequence and assayed as described (14). L-Galactose-1-phosphate phosphatase was cloned from *A. thaliana* (At3g02870, 3.1 μg per assay) and assayed as described (14). GDP-D-mannose 3′,5′-epimerase (EST198296) was cloned from dormant kiwifruit (*A. deliciosa*) buds and assayed as described (35). The substrates of the former two enzymes are highly specific (14, 16).

GDP-L-galactose (≈50% pure, contaminated with the breakdown products GDP and L-galactose-1-phosphate as shown by HPLC and LCMS) and L-galactose-1-phosphate were purchased from Glycoteam. (Hamburg, Germany). We found that GDP-L-galactose was extremely acid labile and did not attempt to purify it further. Other biochemicals were purchased from Sigma (St. Louis, MO).

Activity Assays. The assay for GDP-L-galactose-1-phosphate guanyltransferase was run in 20 mM Tris·Cl, pH 8.0, GDP-L-galactose, with 1 mM D-mannose-1-phosphate. GDP-L-galactose was either used directly from the Glycoteam product (in which case high backgrounds were observed because of contaminating L-galactose-1-phosphate), or GDP-L-galactose was generated by using the epimerase. In the latter case, 0.21 mg of epimerase was incubated with GDP-D-mannose in 20 mM Tris·Cl, pH 8, in a total volume of 400 μl (see ref. 35) for 30 min at 20°C and then used directly in the assay at a 1:20 dilution. Assays were either terminated after 10 min by heating to 100°C for 3 min or directly coupled to the phosphatase and L-galactose dehydrogenase to measure product formation during the assay. Heat-terminated assays were cooled on ice and centrifuged to remove precipitated protein and L-galactose assayed by using the coupling enzymes described above (see also ref. 14). Assays for L-galactose were linear with added L-galactose-1-phosphate over the range measured. Backgrounds were run by using the empty vector control, which gave the same result as a boiled enzyme control.

As an alternative assay, LCMS was used to identify the forward reaction described above as well as to measure the reverse pyrophosphorylase reaction where GTP (1 mM) and L-galactose-1-phosphate were incubated as above and the formation of GDP-L-galactose followed. GDP-D-mannose and GDP-L-galactose were separated by HPLC before MS. LCMS used an LTQ linear ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA) coupled to an Ettan MDLC (GE Healthcare BioSciences). Separation of GDP-D-mannose and GDP-L-galactose was achieved by using a Hypercarb column (ThermoElectron, San Jose, CA), 100 × 2.1 mm, maintained at 40°C. Solvents were (A) 50 mM ammonium acetate and (B) acetonitrile, and the flow rate was 200 μl/min. In the initial mobile phase, 5% B was held for 3 min, then ramped linearly to 20% B at 11 min, held for 5 min, then ramped linearly to 70% B at 19 min and held for 5 min before resetting to the original conditions. Retention times for GDP-D-mannose and GDP-L-galactose were 16.8 and 17.5 min, respectively.

MS data were acquired in the negative mode by using both a selective reaction monitoring (SRM) method SRM m/z 604 > m/z 344, 362, 424, 442, and a selected ion monitoring (SIM) method SIM m/z 604. This SIM method monitors only the (M-H)- ion for GDP-D-mannose and GDP-L-galactose, whereas the SRM method monitors the distinctive daughter ions formed by fragmenting the precursor ion (M-H)- for both compounds. Both methods maximize sensitivity by screening out any chemical noise from other compounds present. The electrospray ionization voltage, capillary temperature, sheath gas pressure, sweep gas, and auxiliary gas were set at −10 V, 350°C, 25 psi, 3 psi, and 3 psi, respectively. Separation of D-mannose-1-phosphate and L-galactose-1-phosphate was achieved isocratically by using a Hypercarb column (ThermoElectron), 100 × 2.1 mm maintained at 40°C. Solvents were (A) 20 mM ammonium acetate and (B) methanol, and the flow rate was 200 μl/min. Using a mobile phase of 2% B, the retention times for D-mannose-1-phosphate and L-galactose-1-phosphate were 4.3 and 4.9 min, respectively. MS data were acquired in the negative mode by using both a SRM method SRM m/z 259 > m/z 79 and 97 and a SIM method SIM m/z 259.

The transferase activity in tobacco leaves was measured by extracting liquid nitrogen ground leaves in ≈5 volumes of Tris·Cl, pH 8.0, 2 mM DTT, and 1 mM EDTA, centrifuging, desalting the supernatant by using a NAP desalting column (GE Healthcare) equilibrated with the same buffer, and assaying the enzyme by using the coupled assay described above. Protein in the extracts was measured by using the Bio-Rad (Hercules, CA) Bradford Coomassie assay (36) by using BSA as a standard.

Transient Transformation of Tobacco Leaves. Tobacco (*Nicotiana benthamiana*) leaves were transiently transformed with *Agrobacterium* containing the EST 319998 cloned in pGreen (37) mixed with *Agrobacterium* containing the gene for the silencing suppressor P19 as described (38). Controls were run by using *Agrobacterium* containing P19 in pGreen alone. Tobacco leaves were harvested 9 days after transformation and frozen in liquid nitrogen.

Ascorbate Measurement. Ascorbate was extracted as described in metaphosphoric acid without reducing agent (39, 40).

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