

L-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient *Arabidopsis* (*vtc*) mutants

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Abstract

Vitamin C (L-ascorbic acid) has important antioxidant and metabolic functions in both plants and animals, humans have lost the ability to synthesize it. Fresh produce is the major source of vitamin C in the human diet yet only limited information is available concerning its route(s) of synthesis in plants. In contrast, the animal vitamin C biosynthetic pathway has been elucidated since the 1960s. Two biosynthetic pathways for vitamin C in plants are presently known. The D-mannose pathway appears to be predominant in leaf tissue, but a D-galacturonic acid pathway operates in developing fruits. Our group has previously shown that transforming lettuce and tobacco with a cDNA encoding the terminal enzyme of the animal pathway, L-gulono-1,4-lactone oxidase (GLOase, EC 1.1.3.8), increased the vitamin C leaf content between 4- and 7-fold. Additionally, we found that wild-type (wt) tobacco plants had elevated vitamin C levels when fed L-gulono-1,4-lactone, the animal precursor. These data suggest that at least part of the animal pathway may be present in plants. To further investigate this possibility, wild-type and vitamin-C-deficient *Arabidopsis thaliana* (L.) Heynh (*vtc*) plants were transformed with a 35S:*GLOase* construct, homozygous lines were developed, and vitamin C levels were compared to those in untransformed controls. Wild-type plants transformed with the construct showed up to a 2-fold increase in vitamin C leaf content compared to controls. All five *vtc* mutant lines expressing GLOase had a rescued vitamin C leaf content equal or higher (up to 3-fold) than wt leaves. These data and the current knowledge about the identity of genes mutated in the *vtc* lines suggest that an alternative pathway is present in plants, which can bypass the deficiency of GDP-mannose production of the *vtc1-1* mutant and possibly circumvent other steps in the D-mannose pathway to synthesize vitamin C.

Abbreviations: AsA, L-ascorbic acid; Gal, L-galactose; GL, L-galactono-1,4-lactone; GLDase, L-galactono-1,4-lactone dehydrogenase; GLOase, L-gulono-1,4-lactone oxidase; GMPase, GDP-D-mannose pyrophosphorylase; GUL, L-gulono-1,4-lactone; Man, D-mannose; wt, wild type

Introduction

The biosynthetic pathways of L-ascorbic acid (vitamin C) differ between plants and animals (Figure 1). In animals, D-glucose is converted to vitamin C via D-glucuronic acid, L-gulonic acid, and L-gulono-1,4-lactone (GUL), which is then oxidized to ascorbate (Loewus, 1963; Smirnoff, 2001). In plants, vitamin C biosynthesis proceeds via GDP-

mannose (GDP-Man), GDP-L-galactose, L-galactose-1-phosphate, L-galactose (Gal) and L-galactono-1,4-lactone (GL) (Wheeler *et al.*, 1998). Although the AsA biosynthetic pathway proposed by Wheeler *et al.* (also called the Smirnoff-Wheeler pathway) is consistent with most available data, there is growing evidence indicating the existence of other pathways operating in plants that contribute to the vitamin C pool. Tracer and feeding studies have shown conversion of methyl-

D-galacturonate and D-glucurono-lactone to vitamin C in detached leaves of several plant species (Loewus, 1963) and *Arabidopsis* cell cultures (Davey *et al.*, 1999). Cloning of a D-galacturonic acid reductase from strawberry (*Fragaria × ananassa* Duch.) fruit, and its expression in *Arabidopsis*, provided molecular evidence for the use of D-galacturonic acid as a precursor for vitamin C (Agius *et al.*, 2003). Recently, we obtained biochemical and molecular data indicating that *myo*-inositol can also serve as precursor for vitamin C biosynthesis in *Arabidopsis* (Lorence *et al.*, 2004).

Evidence supporting the presence of a complex network of metabolic pathways leading to vitamin C comes from the analysis of the vitamin C-deficient *Arabidopsis* (*vtc*) lines (for a historical review, see Conklin, 2001). The first and best characterized of these lines is *vtc1-1*, which retains only 25–30% of the wt vitamin C leaf content. The *VTC1* locus encodes GDP-Man pyrophosphorylase (GMPase, EC 2.7.7.13, mannose-1-phosphate guanyl transferase, step 13; Figure 1), which catalyzes the conversion of mannose-1-phosphate to GDP-Man (Conklin *et al.*, 1999). Other vitamin C-deficient mutants were also isolated and designated as *vtc2-1*, *vtc2-2*, *vtc3-1*, and *vtc4-1* (Conklin *et al.*, 2000). The gene mutated at the *VTC2* locus has been cloned, but the sequence reveals a protein of unknown function (GenBank accession number AF508793; Jander *et al.*, 2002). Activity levels of phosphomannose mutase (step 12, Figure 1), GDP-Man-3,5-epimerase (step 14, Figure 1), Gal dehydrogenase (step 17; Fig. 1) and GL dehydrogenase (GLDase) (step 18; Figure 1) are identical in *vtc2-1*, *vtc3-1*, *vtc4-1* and wt (Smirnoff *et al.*, 2001), leaving only two remaining steps in the D-mannose (Man) pathway where enzyme function could be impaired. Smirnoff *et al.* (2001) have hypothesized that the *VTC2* and *VTC4* loci could be involved in the conversion of GDP-Gal to Gal (steps 15 and 16, Figure 1) and/or in the synthesis of some necessary cofactor or regulatory protein.

In a previous study, a 4- to 7-fold increase in vitamin C content was obtained in lettuce (*Lactuca sativa* L. cultivars Black Seeded Simpson, Grand Rapids and Prizehead) and tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants after constitutive expression of the rat cDNA encoding GLOase, the enzyme involved in the final step of the animal vitamin C biosynthetic pathway. Tobacco plants (wt) also had elevated vitamin C levels when fed GUL, the animal precursor (Jain and Nessler, 2000). Conversion of GUL to vitamin

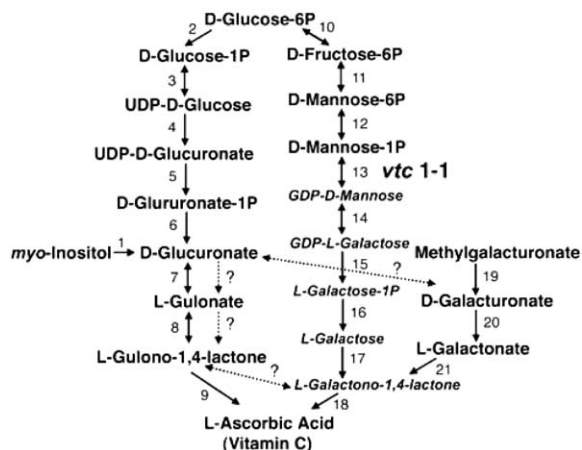


Figure 1. Proposed biosynthetic pathways of L-ascorbic acid in plants (reactions 10–18) and animals (reactions 2–9). Two branch pathways operating in plants, the galacturonic acid pathway and the *myo*-inositol pathway, are also shown. Enzymes catalyzing the numbered reactions are: 1, *myo*-inositol oxygenase; 2, phosphoglucosyltransferase; 3, UDP-glucose pyrophosphorylase; 4, UDP-glucose dehydrogenase; 5, glucuronate-1-phosphate uridylyltransferase; 6, glucuronate-1-phosphate reductase; 7, glucuronate reductase; 8, aldololactonase; 9, gulono-1,4-lactone dehydrogenase; 10, glucose-6-phosphate isomerase; 11, mannose-6-phosphate isomerase; 12, phosphomannomutase; 13, GDP-mannose pyrophosphorylase; 14, GDP-mannose-3,5-epimerase; 15, phosphodiesterase; 16, sugar phosphatase; 17, L-galactose-1-dehydrogenase; 18, L-galactono-1,4-lactone dehydrogenase; 19, methyl-esterase; 20, D-galacturonate reductase; 21, aldono-lactonase. Double-ended arrows indicate reversible enzymatic reactions. Dashed arrows indicate the possible pathway from *myo*-inositol to ascorbic acid and activities of putative epimerases. Smaller font and italics in part of the mannose pathway indicate limiting intermediates for ascorbic acid production in vitamin C-deficient *Arabidopsis* (*vtc*) lines.

C has also been suggested from experiments in cress (*Lepidium sativum* L.; Isherwood *et al.*, 1954), strawberry, and bean (*Phaseolus vulgaris* L.; Baig *et al.*, 1970) where substrate feeding led to an increase in vitamin C level. These data suggest that at least part of the animal vitamin C pathway could be present in plants.

In the present study, wt and vitamin C-deficient *Arabidopsis* (*vtc*) lines were transformed with the rat GLOase cDNA. Homozygous lines of these transformants were generated and vitamin C levels were compared to those in untransformed lines. Wild-type plants expressing GLOase showed up to a 2-fold increase in leaf vitamin C content. All five of the *vtc* mutant lines constitutively expressing the GLOase cDNA had a rescued leaf vitamin C content equal to, or higher (up to 3-fold) than, the wt level. These data, and current knowledge of the identity of the genes

mutated in the *vtc* lines, suggest that one or more alternative vitamin C pathways are present in plants.

Materials and methods

Plant material and growth conditions

Seeds of *A. thaliana* (ecotype Columbia), both wt and homozygous transgenic lines, were grown in Sunshine Mix 1 (Wetzel, Harrisonburg, VA) in a greenhouse during the months of June and July 2003. The greenhouse is equipped with supplemental light (mercury vapor) and a heat-pump to keep temperature and relative humidity conditions as follows: 16/8 h photoperiod, photon flux density of $950 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperature $26/18 \pm 2.5^\circ\text{C}$ (day/night), and relative humidity of $50/70 \pm 10\%$ (day/night).

Construction of transgenic plants

A. thaliana cv. Columbia wt and *vtc* lines were transformed with a 35S:*GLOase* construct (Jain and Nessler, 2000) via the floral dip method (Clough and Bent, 1998). Seedlings were selected on MS (Murashige and Skoog, 1962) plates containing 500 mg l^{-1} carbenicillin and 100 mg l^{-1} kanamycin. Both primary transformants and their progeny were used for RNA gel blot analysis and ascorbic acid assays.

RNA gel blot analysis

Total RNA was extracted from 1 g leaf tissue with the TRI Reagent (Sigma, St. Louis, MO). RNA was suspended in water and precipitated twice with 7 M ammonium acetate and 100% v/v ethanol. RNA yield was quantified spectrophotometrically. For northern analysis, $8 \mu\text{g}$ total RNA was separated on 1.2% w/v denaturing (formaldehyde) agarose gels, and transferred onto nylon membranes (Hybond-N⁺, Amersham, Piscataway, NJ). Membranes were pre-hybridized for 2 h at 65°C and hybridized overnight at 65°C in 500 mM sodium phosphate buffer pH 7.2 containing 7% w/v SDS, 1% w/v bovine serum albumin and 1 mM EDTA. The *GLOase* insert was excised from a pSK:*GLOase* construct (Jain and Nessler, 2000) with *EcoRI*, purified after gel electrophoresis, and labeled with ^{32}P by means of the Primer-It RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA). After hybridization, filters were washed five times for 30 min at 65°C in 20 mM sodium phosphate buffer pH 7.2, 0.1% w/v SDS, 33 mM NaCl, and 1 mM EDTA and subjected to

autoradiography (X-Omat AR film, Kodak, Rochester, NY) between intensifying screens for 16 h at -80°C .

Ascorbic acid measurements

Ascorbic acid content was measured by the ascorbate oxidase assay (Rao and Ormrod, 1995). Plant extracts were made from tissue frozen in liquid nitrogen, ground in 6% w/v meta-phosphoric acid, and centrifuged at $15\,000 \times g$ for 15 min. Reduced ascorbic acid was determined by measuring the decrease in absorbance at 265 nm (extinction coefficient $14.3 \text{ mM}^{-1} \text{ cm}^{-1}$) after addition of 1 U of ascorbate oxidase (Sigma) to 1 ml of the reaction medium containing the plant extract and 100 mM potassium phosphate pH 6.9. Oxidized ascorbic acid was measured in a 1 ml reaction mixture plus 1 μl of 2 mM DTT after incubating at room temperature for 15 min.

Results

All plant lines were grown under identical conditions and rosette leaf tissue samples were taken at the same age and at the same time of day and immediately frozen in liquid nitrogen for later analysis (for details see Materials and methods). These parameters are important because vitamin C values can vary widely with factors such as light conditions (Grace and Logan, 1996; Tabata *et al.*, 2002), time of day, tissue type, and age (Bartoli *et al.*, 2000).

Constitutive expression of GLOase in wild-type plants increases 2-fold leaf vitamin C content

To study the possible contribution of alternative pathways to vitamin C biosynthesis in plants, an ORF encoding the terminal enzyme of the animal pathway, *GLOase*, was expressed under the control of the strong constitutive 35S promoter in wt *A. thaliana* plants. Analysis of four independent homozygous lines (designated L2, L3, L5, and L9) revealed a modest increase (up to 2-fold) in the ascorbic acid content of the leaves compared to untransformed wt plants growing under identical conditions (Figure 2). More than 90% of the ascorbic acid pool was reduced in all plant samples (data not shown). This increase in leaf ascorbic acid content in the over-expressers correlated with the amount of *GLOase* message detected by northern analysis (Figure 2).

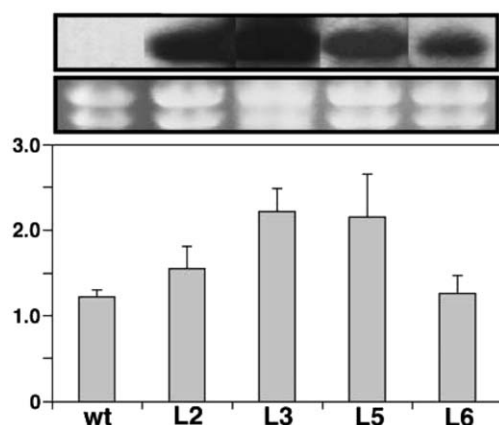


Figure 2. Expression of *GLOase* in *A. thaliana* increases the ascorbic acid content of leaves. Upper panels: expression of *GLOase* RNA in leaves of wild-type (wt) and four homozygous transgenic lines, L2, L3, L5, and L9. Ethidium bromide-stained rRNA shown as loading controls. Lower panels: mean ascorbic acid content ($\mu\text{mol L-AsAgFW}^{-1}$) in the leaf extract of control (wt) and transgenic lines ($n = 3$). Bars represent one standard deviation.

L-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient Arabidopsis (vtc) mutants

To broaden our understanding of the architecture of the network of metabolic pathways leading to vitamin C production, the 35S:*GLOase* construct was also expressed in *Arabidopsis* lines defective in ascorbic acid production. All five *vtc* mutants developed by Dr Patricia Conklin were used in this study. Analysis of three independent homozygous lines (L1, L2, and L3) of each one of the *vtc* mutants revealed a rescued leaf ascorbic acid content as compared to wt and untransformed *vtc* lines growing under identical conditions (Figure 3A–E). As was observed in the wt + *GLOase* plants, more than 90% of the vitamin C pool was reduced in all transformed *vtc* mutants (data not shown). The increase in leaf ascorbic acid content in the transgenics correlated with the amount of *GLOase* message detected by northern analysis (Figure 3A–E).

vtc1-1 plants are smaller than wt plants and show retarded flowering and accelerated senescence (Conklin *et al.*, 1996; Veljovic-Jovanovic *et al.*, 2001). In contrast to the *vtc1-1* line, all three homozygous lines of *vtc1-1* over-expressing *GLOase* grew tall and flowered in a similar fashion to wt (Figure 4A). A similar recovery of the wt growth habit was observed with all other *vtc* mutants constitutively expressing *GLOase* (Figure 4B–E).

Discussion

Constitutive expression of the *GLOase* cDNA resulted in a 2-fold increase in leaf ascorbic acid content of *Arabidopsis* plants compared to controls (Figure 2). We have previously reported a 4- to 7-fold increase in leaf ascorbic acid content in lettuce and tobacco plants expressing the same construct (Jain and Nessler, 2000). Other strategies that have been successful in increasing the leaf ascorbic acid content are the constitutive expression of a D-galacturonic acid reductase from strawberry in *Arabidopsis* (Agius *et al.*, 2003), the over-expression of a *myo*-inositol oxygenase in *Arabidopsis* (Lorence *et al.*, 2004), and the constitutive expression of a dehydroascorbate reductase from wheat (*Triticum aestivum* L.) in tobacco and maize (*Zea mays* L.; Chen *et al.*, 2003). These studies have demonstrated a 2–7-fold increase in the leaf ascorbic acid content of various plant species. In *Arabidopsis*, different strategies consistently result in a 2- to 3-fold increase in leaf ascorbic acid content, possibly reflecting the action of a feedback or other regulatory mechanism that keeps a constant pool of ascorbic acid as has been suggested in previous turnover studies with pea (*Pisum sativum* L.) seedlings (Pallanca and Smirnoff, 2000). As ascorbic acid can become harmful to cells rather than protective when the oxidizing power of the molecule cannot be used (Cabral and Haake, 1988), intermediates, along with ascorbic acid production, may be highly regulated. High concentrations of some intermediates, such as Man, are toxic to plant cells (Harris *et al.*, 1989). *Arabidopsis* cells growing in suspension cultures responded rapidly to high levels of GL and Gal suggesting that the conversion from Man to Gal is tightly controlled (Davey *et al.*, 1999).

Vitamin C-deficient *A. thaliana* (*vtc*) mutants have proven to be a powerful tool in further understanding of ascorbic acid biosynthesis in plants because they provide an opportunity to test proposed biosynthetic pathways. None of these mutants appear to turn over ascorbic acid more rapidly than wt, nor convert D-[U¹⁴C]Man to [¹⁴C]ascorbic acid as efficiently as wt, suggesting that the Smirnoff-Wheeler pathway is diminished in all of them. In the case of *vtc1-1*, it has been clearly demonstrated that the defect in the conversion of both D-glucose and Man to ascorbic acid is due to a point mutation at position +64 of the *gmpase* gene that encodes a Pro-to-Ser change at position 22 of the translated sequence, resulting in substantially decreased GMPase activity, even though the transcript

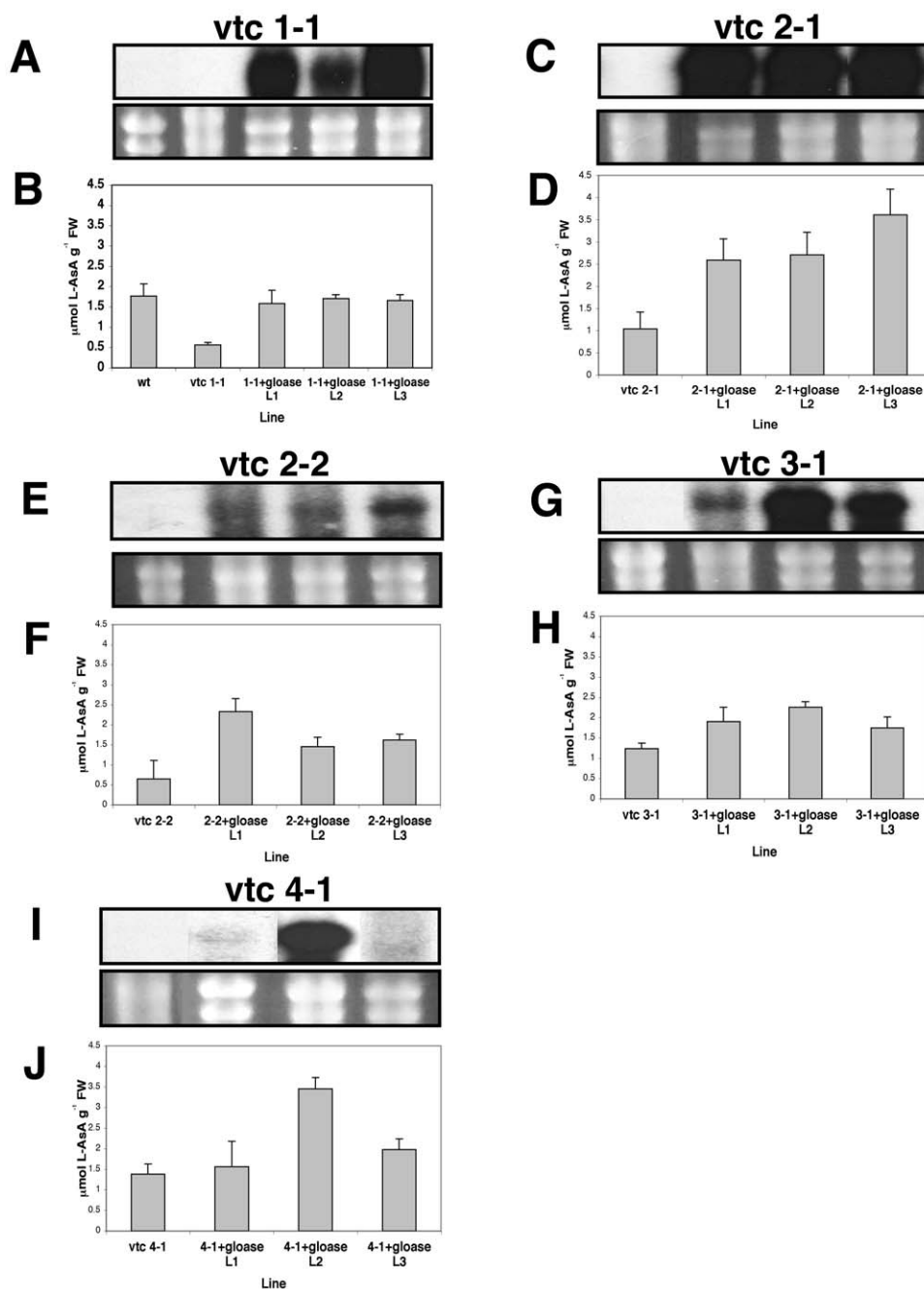


Figure 3. Expression of *GLOase* rescues vitamin C-deficient *A. thaliana* (*vtc*) mutants. A, *vtc1-1*; B, *vtc2-1*; C, *vtc2-2*; D, *vtc3-1*; E, *vtc4-1*. Upper panels: expression of *GLOase* RNA in leaves of transgenic *vtc* mutants. Ethidium bromide-stained rRNA shown as loading controls. Lower panels: mean ascorbic acid content in the leaf extract of control (wt) and transgenic lines ($n = 3$). Bars represent one standard deviation.

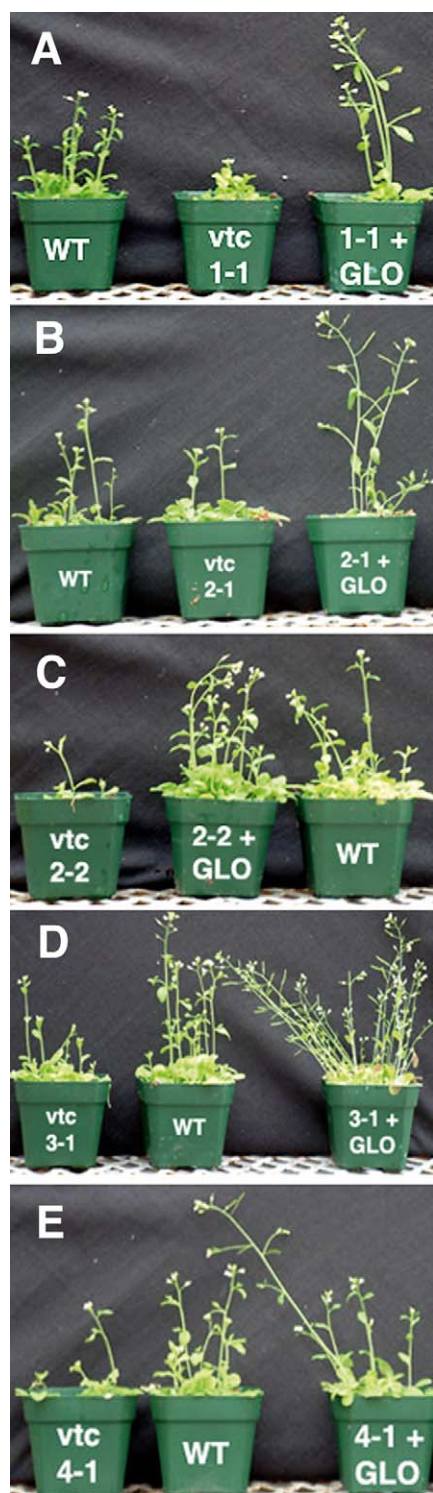


Figure 4. Phenotype rescue of the *vtc* mutants by GLOase. All plants are six weeks old. A, *vtc1-1*; B, *vtc2-1*; C, *vtc2-2*; D, *vtc3-1*; E, *vtc4-1*.

abundance is not affected (Conklin *et al.*, 1996, 1999). All *vtc* mutants, but in particular *vtc1-1*, are good candidates to test the contribution of the Man pathway to ascorbic acid biosynthesis as well as to study the possible contribution of the animal pathway to ascorbic acid production in plants.

As shown in Figure 3, all five *vtc* mutant lines expressing GLOase have a rescued leaf ascorbic content, equal or higher (up to 3-fold) to that of the wt. Expression of GLOase in all *vtc* lines also rescued the phenotype of the mutants: *vtcs* + 35S:*GLOase* grew tall and flowered in a similar fashion to wt plants (Figure 4).

There are two possibilities to explain our observations with wt and *vtc* plants expressing GLOase: (1) the enzyme is working on the Man pathway substrate (GL), or (2) the plants are making the animal pathway precursor (GUL).

Purified GLOase from rat, chicken or kidney is not very specific for its substrate *in vitro* as the protein can also perform the conversion catalyzed by the terminal enzyme of the Man pathway (GLDase) quite efficiently (ca. 85%; Kiuchi *et al.*, 1982 and references therein). GLDase is associated with the inner membrane of mitochondria (Siendones *et al.*, 1999; Bartoli *et al.*, 2000), while the enzyme catalyzing the production of its substrate (Gal dehydrogenase), as well as the other enzymes of the pathway, are located in the cytosol (Gatzek *et al.*, 2002). In the present work, the GLOase cDNA was expressed under the control of the 35S promoter. This promoter causes GLOase to be constitutively expressed at high levels. It is expected that, as in animals, the enzyme is targeted to the ER (Nandi *et al.*, 1997) rather than the mitochondria. Based on the relative low specificity of GLOase and on the differences in localization between GLOase and GLDase, it is possible that the animal enzyme is using the cytosolic fraction of the pool of plant precursor (GL) to produce ascorbic acid. In support of this idea, when the *GLDase* cDNA (containing its mitochondrial signal peptide) from cauliflower (*Brassica oleracea* L. var. *botrytis*; Østergaard *et al.*, 1997) was expressed in tobacco plants the ascorbic acid level of the plants was only increased by ca. 30%, despite a 2- to 3-fold increase in enzymatic activity (Bauw *et al.*, 1998).

The difference in compartmentalization between GLOase and GLDase and the broad specificity of GLOase are possible explanations for our results with the wt *Arabidopsis* plants expressing GLOase, but these differences cannot explain the increased ascorbic acid levels detected in the *vtc1-1* line transformed

with the animal enzyme. The mutation contained in *vtc1-1* causes a diminished GMPase activity and a low production of GDP-Man, a key intermediate of the Smirnov-Wheeler pathway. The production of other intermediates in the pathway that follow GDP-Man – GDP-Gal, Gal-1-phosphate, Gal and GL – appears to be affected as well, as suggested by the observation that the vitamin C deficiency of *vtc1-1* can be reversed by feeding the plants with GL or ascorbic acid (Conklin *et al.*, 1996). This deficiency is not due to alterations in the activity of the terminal enzyme because *vtc1-1* has wt levels of GLDase (Smirnov *et al.*, 2001).

Despite the lack of molecular data regarding the identity of the gene(s) mutated in the other *vtc* lines, all of them appear to be defective in the conversion of Man to ascorbic acid, while they show wt levels in the activities of most of the enzymes in the Man pathway (Smirnov *et al.*, 2001). If the pool of intermediates of the Smirnov-Wheeler pathway is smaller in all *vtc* mutants, as different lines of evidence indicate, the ascorbic acid levels detected in the *vtc* homozygous lines expressing GLOase suggest the presence of an alternative pathway, which can bypass the deficiency of GDP-Man production, and possibly circumvent other steps in the Man pathway.

Another possibility that could explain the results obtained in this work is that plants, both wt and *vtcs*, make GUL, the animal AsA precursor. There is evidence indicating that different plant species have the enzymatic machinery necessary to convert GUL to ascorbic acid (Isherwood *et al.*, 1954; Baig *et al.*, 1970; Davey *et al.*, 1999; Jain and Nessler, 2000). Some authors have suggested that GLDase is able to catalyze the conversion of GUL to ascorbic acid (Smirnov *et al.*, 2001). However, most studies have shown that GLDase is highly (over 99%) specific for GL, as it has been shown for the GLDases purified from spinach (*Spinacia oleracea* L.; Mutsuda *et al.*, 1995), cauliflower (Østergaard *et al.*, 1997), sweet potato (*Ipomoea batatas* L.; Oba *et al.*, 1995; Imai *et al.*, 1998), and *Arabidopsis* (Davey *et al.*, 1999). For both the recombinant and native forms of the tobacco GLDase, GUL was only 7% as effective as a precursor for ascorbic acid compared to GL (Yabuta *et al.*, 2000). In the GUL feeding experiments of Jain and Nessler (2000), the increased levels of ascorbic acid cannot be attributed to the tobacco GLDase when using GUL at a rate of 7%. Others have proposed the presence of a C3-epimerase that could catalyze the inter-conversion of GL and GUL (Baig *et al.*,

1970; Davey *et al.*, 1999). In favor of the idea of the operation in plants of an ascorbic acid pathway that resembles the one from animals, recent metabolic profiling analysis has shown the presence of both galactonic and gulonic acids in *Arabidopsis* (Wagner *et al.*, 2003).

There are several open questions regarding the nature of the pathway(s) from which GLOase is able to 'pull' intermediates for ascorbic acid production. The possible routes that may serve as sources of intermediates for GLOase include: the Man pathway, an undiscovered 'animal pathway', and recently described branch pathways (i.e. the galacturonic acid pathway, Agius *et al.*, 2003; and the myo-inositol pathway, Lorence *et al.*, 2004). The wt + GLOase and *vtc* + GLOase lines described here should be useful tools to help unravel the network of metabolic pathways that lead to ascorbic acid production in plants.

Acknowledgements

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References

- Agius, F., Gonzalez-Lamothe, R., Caballero, J.L., Muñoz-Blanco, J., Botella, M.A. and Valpuesta, V. 2003. Engineering increased vitamin C levels in plants by over-expression of a D-galacturonic acid reductase. *Nature Biotech.* 21: 177–181.
- Baig, M.M., Kelly, S. and Loewus, F. 1970. L-Ascorbic acid biosynthesis in higher plants from L-gulonolactone and L-galactono-1,4-lactone. *Plant Physiol.* 46: 277–280.
- Bartoli, C.G., Pastori, G.M. and Foyer, C.H. 2000. Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol.* 123: 335–343.
- Bauw, G.J.C., Davey, M.W., Østergaard, J. and Van Montagu, M. 1998. Production of ascorbic acid in plants. US Patent 6469149.
- Cabral, J. and Haake, P. 1988. Ascorbic acid 2. Structural determination and synthesis of 2-acyl and 3-acyl derivatives of 5,6-o-isopropylidene-L-ascorbic acid. *J. Org. Chem.* 53: 5742–5750.
- Chen, Z., Young, T.E., Ling, J., Chang, S.C. and Gallie, D.R. 2003. Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proc. Natl. Acad. Sci. USA* 100: 3525–3530.
- Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735–743.
- Conklin, P.L. 2001. Recent advances in the role and biosynthesis of ascorbic acid in plants. *Plant Cell Environ.* 24: 383–394.

- Conklin, P.L., Williams, E.H. and Last, R.L. 1996. Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. *Proc. Natl. Acad. Sci. USA* 93: 9970–9974.
- Conklin, P.L., Norris, S.R., Wheeler, G.L., Williams, E.H., Smirnov, N. and Last, R.L. 1999. Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proc. Natl. Acad. Sci. USA* 96: 4198–4203.
- Conklin, P.L., Saracco, S.A., Norris, S.R. and Last, R.L. 2000. Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. *Genetics* 154: 847–856.
- Davey, M.W., Gilot, C., Persiau, G., Østergaard, J., Han, Y., Bauw, G.C. and Van Montagu, M. 1999. Ascorbate biosynthesis in *Arabidopsis* cell suspension culture. *Plant Physiol.* 121: 535–543.
- Gatzek, S., Wheeler, G.L. and Smirnov, N. 2002. Antisense suppression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis. *Plant J.* 30: 541–553.
- Grace, S.C. and Logan, B.A. 1996. Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. *Plant Physiol.* 112: 1631–1640.
- Harris, G.C., Gibbs, P.B., Ludwig, G., Un, A., Sprengnether, M. and Kolodny, N. 1989. Mannose metabolism in corn and its impact on leaf metabolites, photosynthetic gas exchange, and chlorophyll fluorescence. *Plant Physiol.* 82: 1081–1089.
- Imai, T., Karita, S., Shiratori, G., Hattori, M., Nunome, T., Oba, K. and Hirai, M. 1998. L-Galactono- γ -lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. *Plant Cell Physiol.* 39: 1350–1358.
- Isherwood, F.A., Chen, Y.T. and Mapson, L.W. 1954. Synthesis of L-ascorbic acid in plants and animals. *Biochem. J.* 56: 1–15.
- Jain, A.K. and Nessler, C.L. 2000. Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. *Mol. Breed.* 6: 73–78.
- Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M. and Last R.L. 2002. *Arabidopsis* map-based cloning in the post-genomic era. *Plant Physiol.* 129: 440–450.
- Kiuchi, K., Nishikimi, M. and Yagi, K. 1982. Purification and characterization of L-gulonolactone oxidase from chicken kidney microsomes. *Biochemistry* 21: 5076–5082.
- Loewus, F.A. 1963. Tracer studies on ascorbic acid formation in plants. *Phytochemistry* 2: 109–128.
- Lorence, A., Chevone, B.I., Mendes, P. and Nessler, C.L. 2004. *myo*-Inositol oxygenase offers a possible entry point into plant vitamin C biosynthesis. *Plant Physiol.*, accepted.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* 15: 473–497.
- Mutsuda, M., Ishikawa, T., Takeda, T. and Shigeoka, S. 1995. Subcellular localization and properties of L-galactono-gamma-lactone dehydrogenase in spinach leaves. *Biosci. Biotech. Biochem.* 59: 1983–1984.
- Nandi, A., Mukhopadhyay, C.K., Ghosh, M.K., Chattopadhyay, D.J. and Chatterjee, I.B. 1997. Evolutionary significance of vitamin C biosynthesis in terrestrial vertebrates. *Free Rad. Biol. Med.* 22: 1047–1054.
- Oba, K., Ishikawa, T., Nishikawa, M., Mizuno, H. and Yamamoto, T. 1995. Purification and properties of L-galactono- γ -lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. *J. Biochem.* 117: 120–124.
- Østergaard, J., Persiau, G., Davey, M.W., Bauw, G. and Van Montagu, M. 1997. Isolation of a cDNA coding for L-galactono- γ -lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. *J. Biol. Chem.* 272: 30009–30016.
- Pallanca, J.E. and Smirnov, N. 2000. The control of ascorbic acid synthesis and turnover in pea seedlings. *J. Exp. Bot.* 51: 669–674.
- Rao, M. and Ormrod, D.P. 1995. Ozone pressure decreases UVB sensitivity in a UVB-sensitive flavonoid mutant of *Arabidopsis*. *Photochem. Photobiol.* 61: 71–78.
- Siendones, E., González-Reyes, J.A., Santos-Ocaña, C., Navas, P. and Córbova, F. 1999. Biosynthesis of ascorbic acid in kidney bean. L-galactono- γ -lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. *Plant Physiol.* 120: 907–912.
- Smirnov, N. 2001. L-Ascorbic acid biosynthesis. *Vitam. Horm.* 61: 241–266.
- Smirnov, N., Conklin, P. and Loewus, F.A. 2001. Biosynthesis of ascorbic acid in plants: a renaissance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 437–467.
- Tabata, K., Takaoka, T. and Esaka, M. 2002. Gene expression of ascorbic acid-related enzymes in tobacco. *Phytochemistry* 61: 631–635.
- Veljovic-Jovanovic, S.D., Pignocchi, C., Noctor, G. and Foyer, C.H. 2001. Low ascorbic acid in the *vtc1* mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. *Plant Physiol.* 127: 426–435.
- Wagner, C., Sefkow, M. and Kopka, J. 2003. Construction and application of a mass spectral and retention time index database generated from plant GC/MS metabolite profiles. *Phytochemistry* 62: 887–900.
- Wheeler, G.L., Jones, M.A. and Smirnov, N. 1998. The biosynthetic pathway of vitamin C in higher plants. *Nature* 393: 365–369.
- Yabuta, Y., Yoshimura, K., Takeda, T. and Shigeoka, S. 2000. Molecular characterization of tobacco mitochondrial L-galactono- γ -lactone dehydrogenase and its expression in *Escherichia coli*. *Plant Cell Physiol.* 41: 666–675.