

Antisense suppression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis

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Summary

L-Galactose dehydrogenase (L-GalDH), a novel enzyme that oxidizes L-Gal to L-galactono-1,4-lactone (L-GalL), has been purified from pea seedlings and cloned from *Arabidopsis thaliana*. L-GalL is a proposed substrate for ascorbate biosynthesis in plants, therefore the function of L-GalDH in ascorbate biosynthesis was investigated by overexpression in tobacco and antisense suppression in *A. thaliana*. In tobacco the highest expressing lines had a 3.5-fold increase in extractable activity, but this did not increase leaf ascorbate concentration. *Arabidopsis thaliana*, transformed with an antisense L-GalDH construct, produced lines with 30% of wild-type activity. These had lower leaf ascorbate concentration when grown under high light conditions. L-Gal pool size increased in antisense transformants with low L-GalDH activity, and L-Gal concentration was negatively correlated with ascorbate. The results provide direct evidence for a role of L-GalDH in ascorbate biosynthesis. Ascorbate pool size in *A. thaliana* is increased by acclimation to high light, but L-GalDH expression was not affected. L-Gal accumulation was higher in antisense plants acclimated to high light, indicating that the capacity to synthesize L-Gal from GDP-mannose is increased. Because the only known function of L-GalL is ascorbate synthesis, these antisense plants provide an opportunity to investigate ascorbate function with minimal effects on carbohydrate metabolism. Measurements of other antioxidants revealed an increase in ascorbate- and pyrogallol-dependent peroxidase activity in low-ascorbate lines. As ascorbate is the major hydrogen peroxide-scavenging antioxidant in plants, this could indicate a compensatory mechanism for controlling hydrogen peroxide concentration.

Keywords: antisense suppression, *Arabidopsis thaliana*, ascorbate, L-galactose, L-galactose dehydrogenase, peroxidase, tobacco, transgenic plant.

Introduction

Although ascorbate is a major plant constituent and is important in human nutrition, a biosynthetic pathway via GDP-mannose (GDP-Man) and L-galactose (L-Gal) was proposed only recently (Smirnoff *et al.*, 2001; Wheeler *et al.*, 1998). GDP-Man is first converted to GDP-L-Gal by GDP-Man-3,5-epimerase. GDP-mannose-3,5-epimerase was first identified in *Chlorella* (Barber, 1979; Hebda *et al.*, 1979), pea and *A. thaliana* (Wheeler *et al.*, 1998),

and has now been cloned from *A. thaliana* (Wolucka *et al.*, 2001). L-Gal is then formed from GDP-L-Gal by as yet uncharacterized steps. L-Gal is oxidized to L-galactono-1,4-lactone (L-GalL) by L-galactose dehydrogenase (L-GalDH) and then to ascorbate by L-galactono-1,4-lactone dehydrogenase, an enzyme located on the inner mitochondrial membrane (Bartoli *et al.*, 2000; Siendones *et al.*, 1999). GDP-Man is synthesized from Man-1-P by GDP-Man

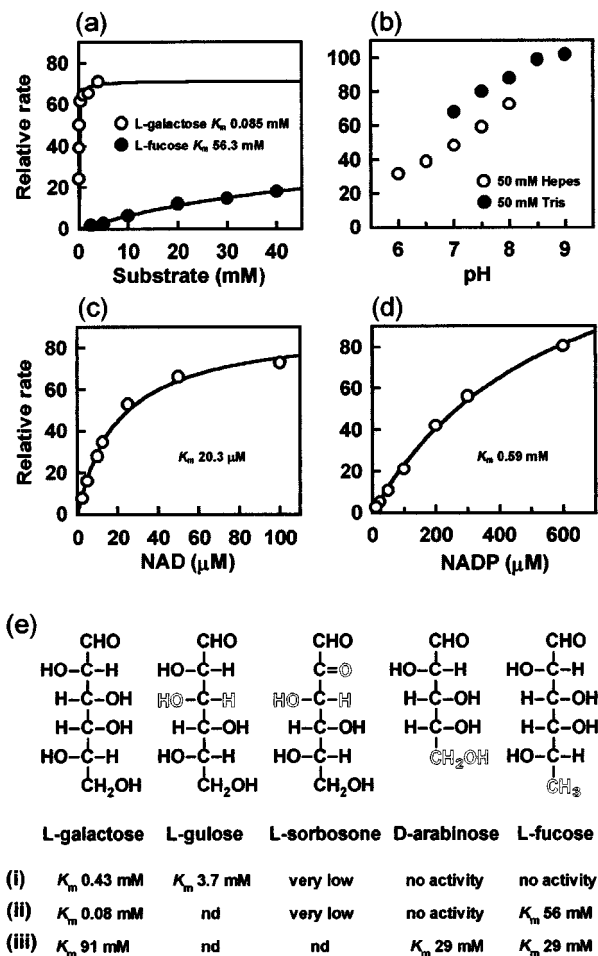


Figure 1. Properties of L-galactose dehydrogenase. (a–d) Affinity for L-galactose and L-fucose (a); pH dependence of L-galactose oxidation (b); and affinity for cofactors NAD⁺ (c) and NADP⁺ (d) of recombinant *A. thaliana* L-galactose dehydrogenase. (e) Comparison of the substrate specificity of aldose dehydrogenases. Structural differences of other potential substrates compared to L-galactose are highlighted. K_m values of (i) pea L-galactose dehydrogenase; (ii) recombinant *A. thaliana* L-galactose dehydrogenase; and (iii) yeast D-arabinose dehydrogenase (Kim *et al.*, 1996; Kim *et al.*, 1998) for L-galactose and structurally related carbohydrates are indicated; nd, not determined.

pyrophosphorylase. The ascorbate-deficient *A. thaliana* mutant *vtc1* has low GDP-Man pyrophosphorylase activity (Conklin *et al.*, 1999). Furthermore, potato plants expressing antisense GDP-Man pyrophosphorylase have low ascorbate and, because GDP-Man is also a substrate for polysaccharide synthesis, lower mannose content in their cell walls (Keller *et al.*, 1999). The discovery of L-GalDH, a novel and unique enzyme in plants which oxidizes C1 of L-Gal to form L-GalL, was key to the proposal of the pathway (Wheeler *et al.*, 1998). Although relatively little is known about L-Gal metabolism in comparison to the more abundant D-Gal, its occurrence in plant cell walls and algal and mollusc polysaccharides has been known for

some time (Feingold, 1982). Plant cell wall polysaccharides also contain L-fucose (6-deoxy-L-Gal) which is formed from GDP-Man. *Mur1* mutants deficient in GDP-Man-4,6-dehydratase are unable to synthesize GDP-L-fucose, and have increased L-Gal content in their wall polysaccharides, presumably as a result of increased GDP-L-Gal availability (Bonin *et al.*, 1997; Reiter *et al.*, 1993).

Ascorbate has a multitude of proposed functions in plants. It is an antioxidant, providing protection against oxidative stress by scavenging hydrogen peroxide and other reactive oxygen species and by regenerating the membrane-localized antioxidant α -tocopherol (Asada, 1999; Noctor and Foyer, 1998; Smirnov, 2000b). It is an enzymatic cofactor for violaxanthin de-epoxidase (Neubauer and Yamamoto, 1994) and a variety of Fe-dependent dioxygenases (Arrigoni and De Tullio, 2000; Davey *et al.*, 2000; De Tullio *et al.*, 1999). Together with ascorbate oxidase, it could be involved in cell expansion and cell division (Kato and Esaka, 1999; Kato and Esaka, 2000). At least in some plant species, ascorbate is used as substrate for oxalate and tartrate synthesis (Keates *et al.*, 2000; Kostman *et al.*, 2001; Loewus, 1999). Transgenic plants and mutants have provided evidence for these functions. Tobacco BY-2 cell cultures expressing an antisense L-galactono-1,4-lactone dehydrogenase (L-GalLDH) have 70% of wild-type ascorbate and exhibit a reduction in cell growth rate as well as abnormal cell shape (Tabata *et al.*, 2001). The low-ascorbate *A. thaliana* mutant *vtc1* (which has low GDP-mannose pyrophosphorylase activity) is smaller than the wild type (Veljovic-Jovanovic *et al.*, 2001). Low-ascorbate potato plants expressing antisense GDP-mannose pyrophosphorylase (Keller *et al.*, 1999) are smaller and the leaves senesce more rapidly. However, in the case of these plants the effect of ascorbate deficiency cannot be distinguished from other effects of perturbing mannose metabolism. The *vtc* mutants are generally more sensitive to ozone and photo-oxidative stress (Conklin, 2001; Conklin *et al.*, 1996; Conklin *et al.*, 2000; Smirnov, 2000b).

It is clear that ascorbate has a number of roles in stress resistance, growth and control of hydrogen peroxide concentration through ascorbate peroxidase (APX) activity (Noctor and Foyer, 1998; Smirnov, 2000a). However, there is a need for more investigations, both into the proposed ascorbate biosynthetic pathway and to establish the importance of these proposed roles. In this paper we report on the characterization and cloning of *A. thaliana* L-GalDH, and describe the results of its overexpression and antisense suppression in transgenic plants. The transgenic plants were used to confirm the role of L-GalDH in ascorbate biosynthesis, to reveal the synthesis of free L-Gal in plants and its control by light. We discuss the potential of using these plants, which are minimally

affected in other aspects of carbohydrate metabolism, to investigate the functions of ascorbate.

Results

Purification and properties of pea L-GalDH

Embryonic axes from germinating pea seedlings, which are very active in ascorbate synthesis (Pallanca and Smirnof, 1999; Pallanca and Smirnof, 2000), have high L-GalDH activity. L-GalDH was purified from embryonic axis extracts by ammonium sulphate precipitation (50–70% saturation), hydrophobic interaction chromatography, anion exchange chromatography, Blue Sepharose affinity chromatography and gel filtration. The molecular weight of L-GalDH was estimated by gel filtration to be 156 kDa, while the subunit molecular weight was 40 kDa, suggesting that the enzyme is a homotetramer. The 40 kDa band was separated by SDS-PAGE and the N-terminal sequence was determined (AELRELGRTGLKGLVGF). We have previously shown that L-Gal is oxidized at C1 by L-GalDH, the product being L-GalL (Wheeler *et al.*, 1998). It has a K_m of 0.43 mM for L-Gal and is also active with L-gulose ($K_m = 3.7$ mM; Figure 1). Activity with 0.1 mM NADP⁺ is 6% of that NAD⁺ and it has a pH optimum of 8. Activity was inhibited by 90% with 1 mM *N*-ethylmaleimide (NEM) and by 65% with 5 mM oxidized glutathione, suggesting involvement of a thiol group. The reaction is not reversible, but is inhibited in an uncompetitive manner by its product L-GalL.

Cloning and functional characterization of Arabidopsis thaliana L-GalDH

A BLAST search for similarities to the N-terminal amino acid sequence of the pea L-GalDH found one *A. thaliana* gene encoding a putative protein (Accession No. CAA20580) of unknown function. The corresponding cDNA was cloned by RT-PCR. Addition of a His tag and expression of this cDNA in *Escherichia coli* resulted in a protein with L-GalDH activity, which was purified by affinity chromatography and gel filtration. The recombinant protein has a molecular weight of 42.2 kDa, determined by SDS-PAGE, and two peaks of activity at 42.4 and 87.5 kDa by gel filtration, indicating that its native form is a homodimer. The recombinant L-GalDH has a K_m of 0.085 mM for L-galactose, but also oxidizes L-fucose and L-sorbose at lower rates (Figure 1). No activity was detectable with D-arabinose, D-galactose, D-glucose and D-mannose. The preferred cofactor is NAD⁺ (Figure 1), and the pH optimum is between 8.5 and 9. Southern blot analysis shows that L-GalDH is encoded by a single gene in *A. thaliana*.

L-GalDH activity and expression in Arabidopsis thaliana and other species

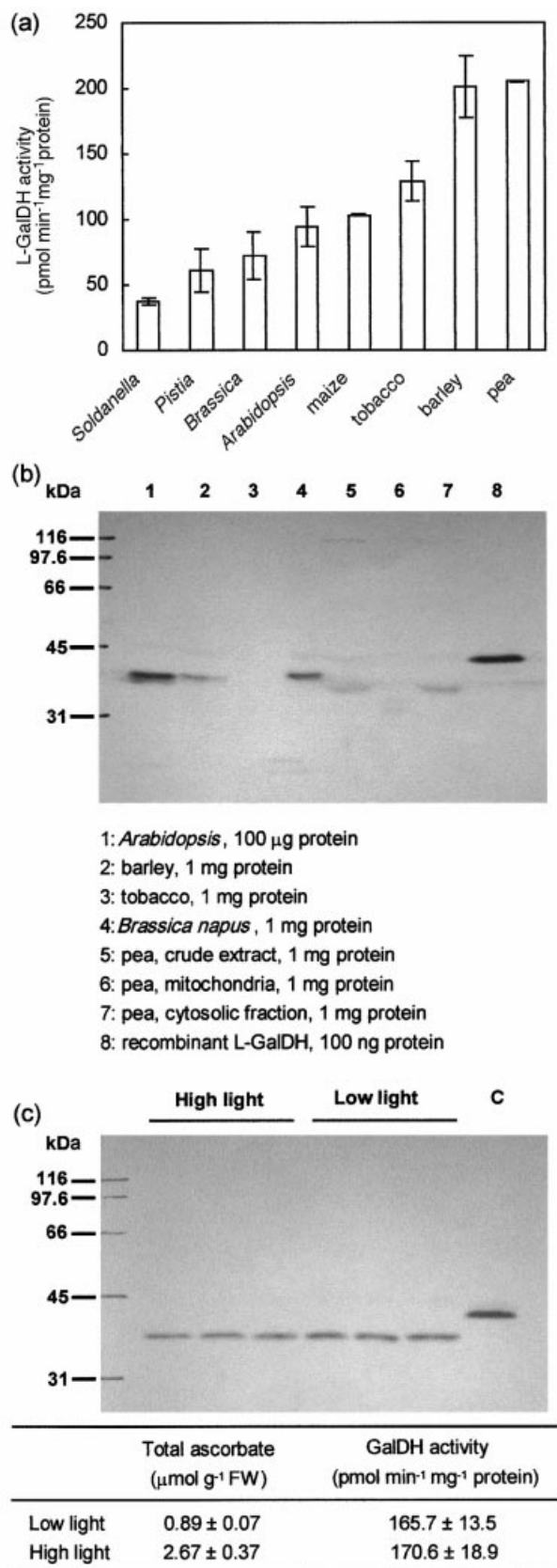
L-GalDH activity varied between 50 and 200 pmol min⁻¹ per mg protein in various species (Figure 2a). A polyclonal antibody raised against recombinant L-GalDH recognizes the *A. thaliana* and *Brassica napus* proteins, but cross-reacted with other species less effectively (Figure 2b). Ascorbate-deficient *Arabidopsis vtc* mutants (Conklin *et al.*, 2000) did not show significant differences in L-GalDH transcript level, immunodetectable protein or L-GalDH activity (data not shown). No L-GalDH was detectable in washed mitochondrial preparations of pea seedlings by activity assay or Western blotting (Figure 2b), although it was detected in the crude extract and supernatant. This suggests that L-GalDH is not mitochondrial. Ascorbate pool size in *A. thaliana* is higher at high light intensity, but neither L-GalDH activity nor immunodetectable protein were affected in plants acclimated to low or high light (Figure 2c).

Overexpression of L-GalDH in tobacco has no effect on foliar ascorbate concentration

Tobacco was transformed with *A. thaliana* L-GalDH driven by the CaMV 35S promoter and with an empty binary vector as control (Figure 3a). L-GalDH activity in 20 independent lines (T_1 progeny) varied from unaffected to a 3.5-fold increase in the highest expressing lines compared to wild-type and vector control transformants (Figure 3c). The concentrations of L-GalDH transcript and protein corresponded to activity (Figure 3b). Although L-GalDH activity was effectively increased, there was no effect on ascorbate concentration in leaves of the transformants (Figure 3d). L-Gal is rapidly converted to ascorbate by *A. thaliana* leaves, resulting in increased pool size, so this assay was used to assess if the overexpressed enzyme is active *in vivo* in leaf slices of the two highest expressing lines (GDH 21 and 36). The accumulation of ascorbate from exogenous L-Gal was only slightly greater than the empty vector control plants, and was not visible until 24 h incubation (Figure 3e). This small increase in L-Gal conversion compared with non-overexpressing leaves was much less than the 3.5-fold increase in extractable enzyme activity.

Antisense suppression of L-GalDH activity in Arabidopsis thaliana reduces foliar ascorbate concentration

Arabidopsis thaliana was transformed with an antisense construct of the L-GalDH gene (Figure 4a). Southern blot analysis revealed a single transgene copy in most of the antisense lines except for anti-GDH 18 and 32 with two copies, and anti-GDH 42 with three copies, of antisense L-

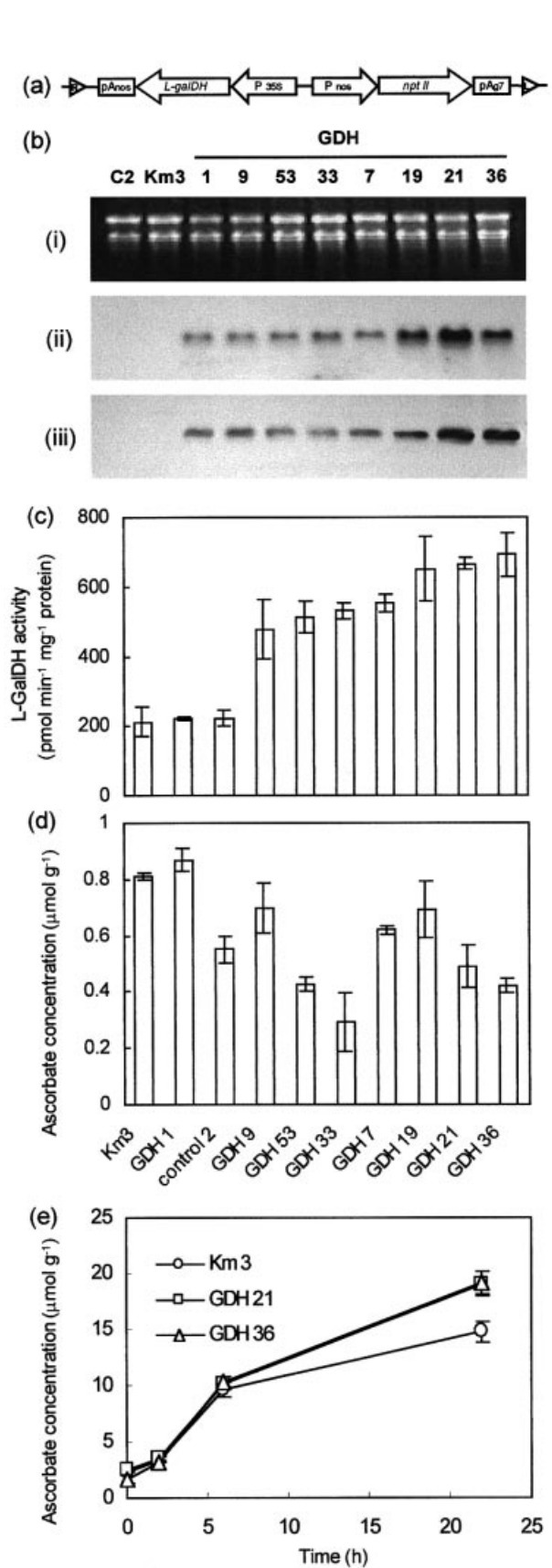


GalDH. The lines with high antisense transcript abundance had less immunodetectable L-GalDH protein than the controls (Figure 4b). Ascorbate pool size in *A. thaliana* is increased at high light intensity (Figure 2c), so the effect of antisense suppression of L-GalDH was investigated under low and high light intensity. L-GalDH activity and leaf ascorbate concentration were initially determined in plants grown under relatively low light intensity (photosynthetic photon flux density 100 µmol m⁻² sec⁻¹), and then 4 days after transferring the plants to higher light intensity (photosynthetic photon flux density 400 µmol m⁻² sec⁻¹). Lines with reduced L-GalDH expression (Figure 4b) had reduced L-GalDH activity to a minimum of 30% of wild type in the lines with strongest suppression (Figure 4c). Light intensity had no effect on L-GalDH activity (Figure 4c). At low light intensity the smaller ascorbate pool was unaffected by L-GalDH activity in five transformed lines, but at high light intensity the ascorbate concentration in plants with low L-GalDH was lower, reaching 45% of the wild-type level in anti-GDH 42 (Figure 4d). Leaf slices from antisense lines with reduced L-GalDH activity also had a reduced capacity to convert exogenous L-galactose to ascorbate (Figure 4e), whereas they were able to convert L-galactono-1,4-lactone, the product of GalDH activity, to ascorbate at the same rate as wild-type plants (Figure 4f). The anti-GDH plants showed no growth reduction or other visible phenotype, although they were generally grown at low light intensity which minimizes the effect on ascorbate.

Antisense suppression of L-GalDH causes light-dependent L-galactose accumulation

The recombinant L-GalDH provides a sensitive and selective tool for determining L-Gal concentration in an NAD⁺-linked fluorimetric assay. D-Gal is not detected, although L-fucose would also be detected (Figure 1) but its concentration in *A. thaliana* is extremely low (N.S., unpublished GC and GC-MS results). The assay was used to detect L-Gal in wild-type and transgenic *A. thaliana* with reduced L-GalDH activity. In wild-type plants, L-Gal concentration is very near the detection limit of the assay (2 nmol L-Gal g⁻¹ FW) but it was easily detected in three anti-GDH lines with low L-GalDH activity (Figure 5a). The results show that plants can synthesize free L-Gal. The increased L-Gal concentration in the leaves correlated negatively with the

Figure 2. L-Galactose dehydrogenase expression in several plant species. (a) L-GalDH activity; (b) expression of L-GalDH in different species and cellular compartments; (c) Western blot analysis, L-GalDH activity and ascorbate concentration in *Arabidopsis* grown under low and high light. Each lane of the SDS-PAGE (c) was loaded with 80 µg protein, except lane C, loaded as control with 100 ng recombinant L-GalDH, which is larger than the plant protein due to a (His)₆-tag.



ascorbate concentration in these lines, as would be expected if L-Gal is the precursor of ascorbate *in vivo* (Figure 5b).

The relationship between L-Gal accumulation and ascorbate biosynthesis was investigated further by comparing the L-Gal concentration in leaves of two antisense lines acclimated to low and high light (Figure 5c). In anti-GDH 18, a line with intermediate L-GalDH reduction and L-Gal accumulation, light had no effect. However, in anti-GDH 42, which has the greatest reduction in L-GalDH activity and the largest L-Gal accumulation, more L-Gal accumulated in plants acclimated to high light for 4 days. Short-term transfer (4 h) of detached leaves from low light-acclimated plants to high light did not increase L-Gal, while leaves transferred from high light-acclimated plants to low light still maintained a larger L-Gal pool than low light-acclimated plants. Therefore, reduction of L-GalDH activity has revealed that L-Gal synthesis is increased by acclimation to high light.

Pleiotropic effects of reduced L-GalDH activity on other antioxidants

Because reduced ascorbate content is expressed only at high light intensity, the effect of antisense suppression of L-GalDH on other aspects of the antioxidant system was investigated in plants acclimated to high light for 4 days. No significant differences could be detected in α-tocopherol concentration or in the activities of monodehydroascorbate reductase, glutathione-dependent dehydroascorbate reductase and glutathione reductase (data not shown). Glutathione concentration tended to be lower in antisense plants with low ascorbate (Figure 6a). The concentration of oxidized glutathione was 5% of the total, and constant in all lines. Peroxidase activity was increased in the low-ascorbate lines. This was true for total

Figure 3. Overexpression of *L-GalDH* in tobacco. (a) T-DNA of the vector pGPTV-LGalDH. *L-GalDH*, *Arabidopsis thaliana* L-galactose dehydrogenase gene; *npt II*, neomycin phosphotransferase gene; P_{35S}, CaMV 35S promoter; P_{nos}, nopaline synthase promoter; pAnos and pAg7, nopaline synthase and gene 7 poly(A) signals; R and L, right and left T-DNA borders. (b) Expression of *A. thaliana* L-galactose dehydrogenase in tobacco (i) ethidium bromide stained agarose gel loaded with 10 μg lane⁻¹ total RNA from tobacco SRI (C2), a vector control (Km3) and 8 individual GDH lines (ii) Northern blot probed with *A. thaliana* L-GalDH (iii) Western blot from SDS-PAGE loaded with 50 μg protein per lane. (c) L-Galactose dehydrogenase activity and (d) ascorbate concentration in wild type tobacco SRI (control 2), a vector control line (Km3) and 8 individual GDH lines, which were transformed with the *A. thaliana* L-galactose dehydrogenase gene. (e) *In vivo* conversion of L-galactose to ascorbate in tobacco. Leaf slices (2 mm wide) of a vector line (Km3) and two *A. thaliana* L-galactose dehydrogenase overexpressing lines (GDH 21 and GDH 36) were incubated with 10 mM L-galactose under a photon flux density of 300 μmol m⁻² sec⁻¹.

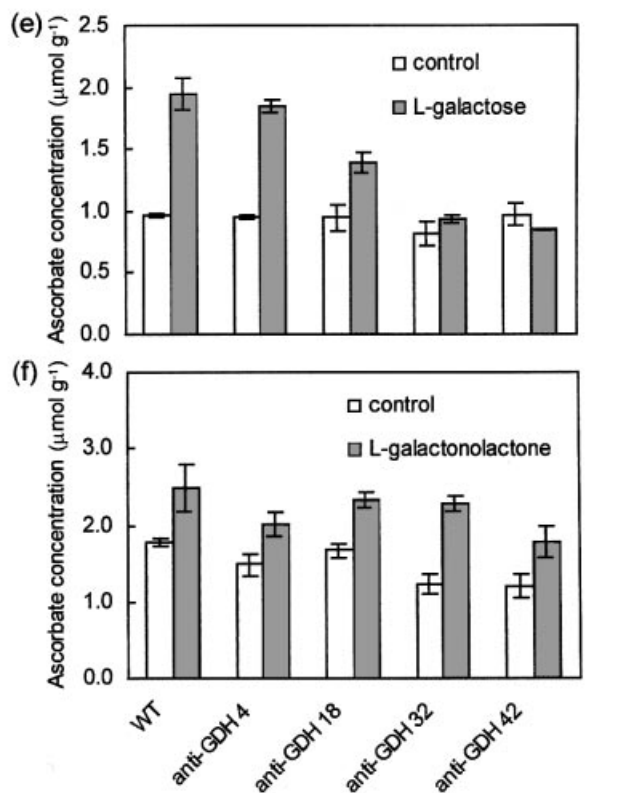
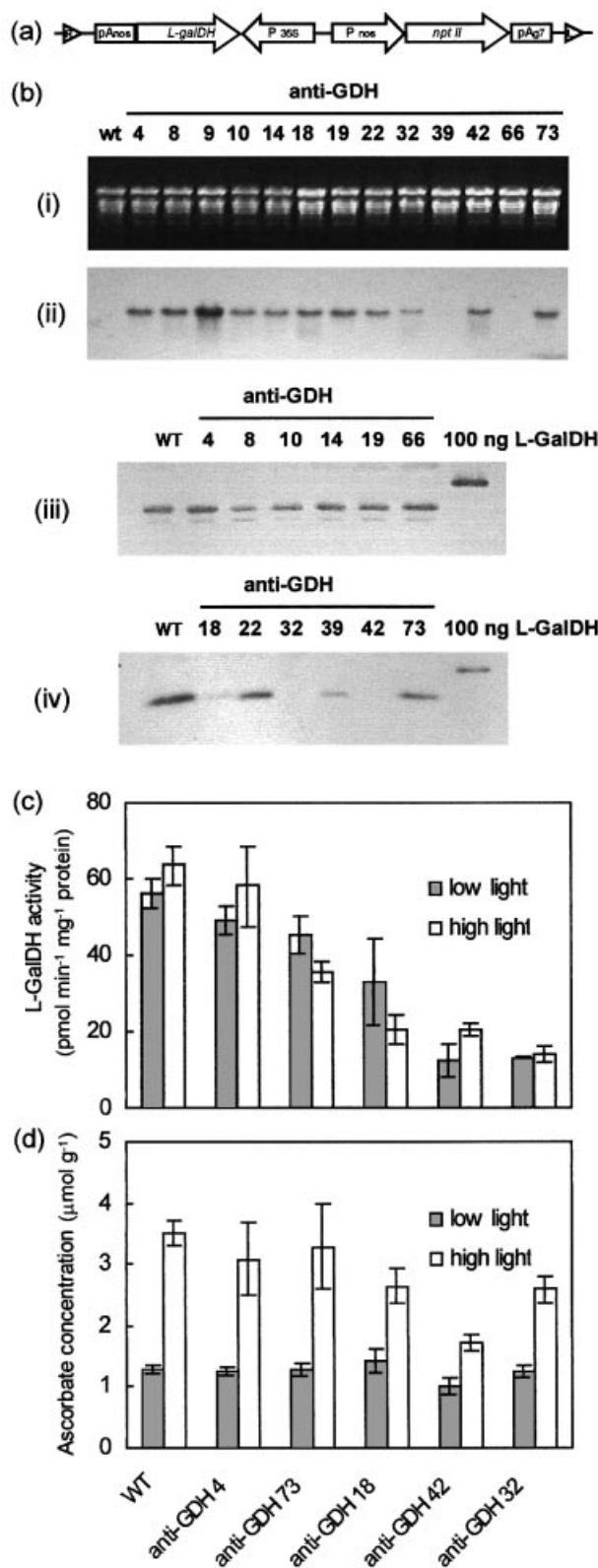


Figure 4. Antisense suppression of *L-GalDH* in *Arabidopsis*.

(a) T-DNA of the vector pGPTV-anti-LGalDH. *L-galDH*, *A. thaliana* *L*-galactose dehydrogenase gene. *npt II*, neomycin phosphotransferase gene; *P35S*, CaMV 35S promoter; *Pnos*, nopaline synthase promoter; *pAnos* and *pAg7*, nopaline synthase and gene 7 poly(A) signals; R and L, right and left T-DNA borders.

(b) Expression of *L*-galactose dehydrogenase in *Arabidopsis* antisense transformants: (i) ethidium bromide-stained agarose gel loaded with 10 μg per lane total RNA from wild-type (WT) and 13 individual anti-GDH lines; (ii) Northern blot probed with *A. thaliana* *L-galDH*; (iii) Western blot from SDS-PAGE loaded with 80 μg protein per lane and 100 ng recombinant *L*-galactose dehydrogenase as control.

(c,d) *L*-Galactose dehydrogenase activity (c) and ascorbate concentration (d) in *Arabidopsis* wild-type (WT) and five antisense lines.

(e,f) *In vivo* conversion of ascorbate precursors. Leaf slices (2 mm wide) of wild-type (WT) and four antisense *L*-galactose dehydrogenase lines were incubated for 6 h with 10 mM *L*-galactose, 10 mM *L*-galactonolactone and water as control under a photon flux density of 300 μmol m⁻² sec⁻¹.

soluble ascorbate peroxidase activity (Figure 6b) and peroxidase activity measured with pyrogallol as substrate (Figure 6c).

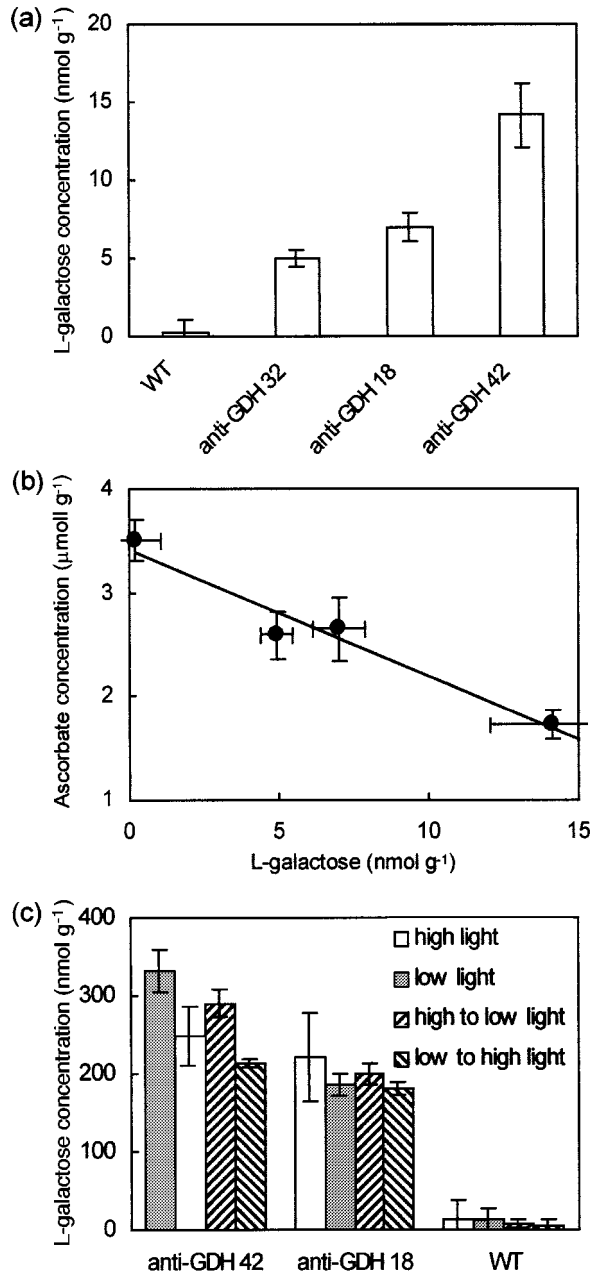


Figure 5. Accumulation of L-galactose in antisense L-GalDH *Arabidopsis*. (a,b) L-Galactose concentration (a) and the relationship between ascorbate and L-galactose concentration (b) in wild-type (WT) and antisense L-galactose dehydrogenase lines.

(c) Accumulation of L-galactose under different light conditions. Wild-type *Arabidopsis* (WT) and the antisense L-galactose dehydrogenase lines 42 and 18 were acclimatized to high light ($400 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and low light ($50 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and incubated under reversed conditions for 4 h.

Discussion

Characteristics of L-GalDH

L-GalDH activity has been detected in all the plant species we have examined. However, at the present time no potential orthologues of the *A. thaliana* GalDH gene have been identified in other plants. The poor cross-reactivity of the *A. thaliana* L-GalDH polyclonal antibody with other species could indicate appreciable sequence divergence. The closest similarity (30% identity) is to L-fucose (6-deoxy-L-galactose) dehydrogenase from *Pseudomonas* (Yamamoto-Otake *et al.*, 1994). This enzyme has K_m values of 1.9 mM, 19 mM and 300 mM for L-fucose, L-Gal and D-

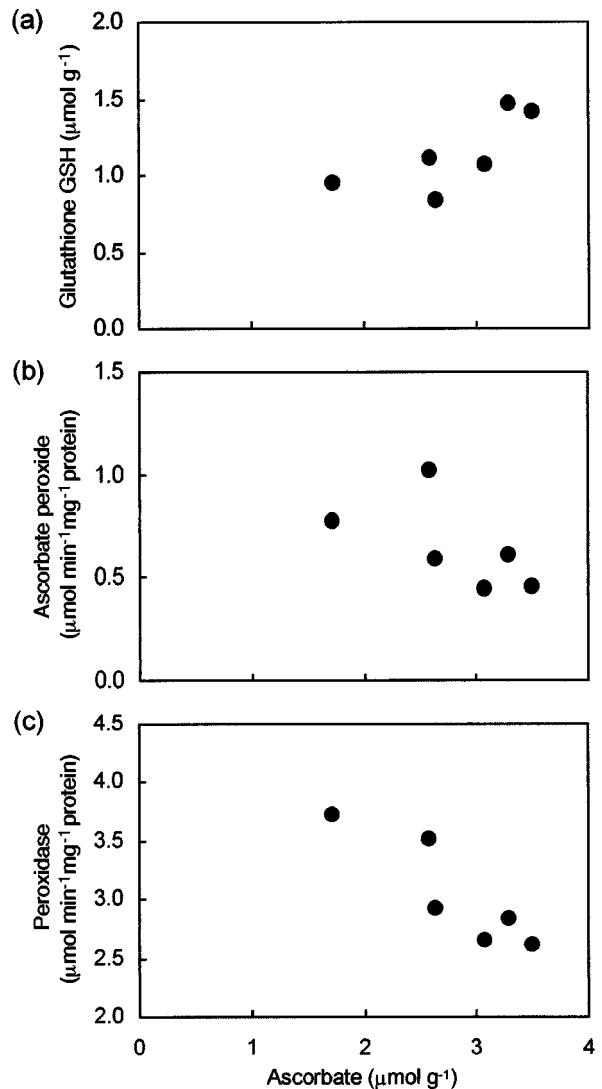


Figure 6. Effects of antisense suppression of L-galactose dehydrogenase in *A. thaliana* on (a) glutathione concentration; (b) ascorbate peroxidase activity; and (c) pyrogallol peroxidase activity in relation to ascorbate concentration in six individual lines.

arabinose, respectively, and prefers NADP⁺ to NAD⁺ (Horiuchi *et al.*, 1989); it therefore has a different substrate preference to L-GalDH. Other than oxidoreductases from micro-organisms, no similar eukaryotic genes have been identified, although L-fucose dehydrogenase activity is reported from liver (Schachter *et al.*, 1969) and yeast (Conter *et al.*, 1984). In plants, as far as we are aware, no other dehydrogenase able to oxidize non-phosphorylated sugars has been reported. The sequence of L-GalDH is similar to other members of the aldo/ketose reductase family which includes *Pseudomonas* L-fucose dehydrogenase. This also accounts for its similarity (30% identity) to the β -chain regulatory domain of redox-regulated potassium channels. Yeast synthesizes D-erythroascorbate (a 5C analogue of L-ascorbate) instead of ascorbate, by a pathway analogous to the plant pathway. D-Arabinose is oxidized by D-arabinose dehydrogenase at C1 forming D-arabinono-1,4-lactone, which is then oxidized to erythroascorbate (Kim *et al.*, 1996, 1998). D-arabinose dehydrogenase from *Saccharomyces cerevisiae*, like the plant and *Pseudomonas* enzymes, also catalyses L-fucose and L-Gal oxidation, but is entirely unrelated in amino acid sequence to the *A. thaliana* and *Pseudomonas* enzymes (Kim *et al.*, 1998; Yamamoto-Otake *et al.*, 1994).

L-GalDH is a soluble enzyme with no obvious targeting or transit sequences. It was not detected in pea mitochondria, and it is most likely to be a cytosolic enzyme. The final enzyme in the pathway, L-galactono-1,4-lactone dehydrogenase (L-GalLDH), which oxidizes L-GalL to ascorbate, is located on the outer side of the inner mitochondrial membrane (Bartoli *et al.*, 2000; Siendones *et al.*, 1999), so L-GalL would be transported from the cytosol into the intermembrane space. The simple kinetic characteristics suggest that GalDH has no regulatory properties. Pea and *A. thaliana* L-GalDH have similar subunit sizes, although it is a homotetramer in pea and a homodimer in *A. thaliana*. It is not clear if this is a real difference or if it is caused by the (His)₆-tag attached to the recombinant *A. thaliana* enzyme.

The role of L-GalDH in ascorbate biosynthesis

The manipulation of GalDH in transgenic plants has provided the first direct evidence that this enzyme, as originally proposed by Wheeler *et al.* (1998), is actually involved in ascorbate biosynthesis. This adds to other genetic evidence supporting the Smirnov–Wheeler pathway of ascorbate biosynthesis from GDP-mannose via GDP-L-Gal, L-Gal and L-GalL (Smirnov *et al.*, 2001; Wheeler *et al.*, 1998). First, the *A. thaliana vtc1* mutant (Conklin *et al.*, 1999) and antisense potatoes (Keller *et al.*, 1999) have reduced GDP-mannose pyrophosphorylase activity and reduced ascorbate content. Second, tobacco BY-2 cells in which L-GalLDH activity was reduced by antisense sup-

pression have lower ascorbate (Tabata *et al.*, 2001). Alternative biosynthetic pathways have been suggested in which L-GalL (formed from D-galacturonate) or L-gulonono-1,4-lactone (formed from D-glucuronate) could be ascorbate precursors (Davey *et al.*, 1999; Loewus, 1999). Their contribution could be tested by elimination of L-GalDH activity as neither alternative pathway requires L-Gal. Unfortunately, we were unable to reduce activity far enough by antisense suppression to test this possibility. It should be possible to use knockout mutants of L-GalDH to investigate ascorbate synthesis and function because null mutants, which are potentially lethal, could be rescued by exogenous GalL.

Two observations suggest that L-GalDH activity exerts relatively little control over flux through the pathway. First, overexpression of L-GalL in tobacco had no effect on ascorbate concentration. Second, at low light intensity, when ascorbate pool size (and presumably pathway flux; see next section) is low, reduced L-GalDH activity has no effect on ascorbate. Reduced expression of L-GalDH did decrease ascorbate under high light intensity, but there was a proportionately smaller reduction in ascorbate than in L-GalDH activity. This suggests that L-Gal will begin to exert an effect at very high flux. The very low L-Gal concentration detected in wild-type plants also suggests that the capacity of L-GalDH to use L-Gal is much higher than its rate of synthesis. The spare capacity of L-GalDH is also indicated by the rapid increase in ascorbate pool size caused by exogenous L-Gal (Figure 4; Smirnov, 2000b; Wheeler *et al.*, 1998). However, it is interesting that the increased L-GalDH activity in leaf extracts of tobacco overexpressing lines was not matched by a proportionate increase in conversion of exogenous L-Gal to ascorbate (Figures 3c,e). This could be caused, for example, by L-Gal uptake rate or NAD⁺ supply becoming limiting.

Light increases ascorbate and L-Gal synthesis

The ascorbate pool size in *A. thaliana* is affected by light. It increases when plants are acclimated to higher light (Figures 2 and 4) over a period of 2–3 days (J. Dowdle, University of Exeter, Exeter, UK and N.S., unpublished results). Conversely, the pool size decreases if leaves are kept in the dark (Conklin *et al.*, 1997). Similar light responses occur in leaves of barley and other species (Smirnov, 2000b; Smirnov and Pallanca, 1996). It is likely that these responses are related to the proposed roles of ascorbate in protection against photo-oxidative stress (Noctor and Foyer, 1998; Smirnov, 2000b). While it is probable that increased pool size is caused by increased synthesis, the use of labelled precursors to compare flux at low and high light intensity has provided equivocal results (N.S., unpublished results). Also, ascorbate turnover is appreciable (Pallanca and Smirnov, 2000) and could

control pool size. Greater accumulation of L-Gal in the anti-GDH 42 line after acclimation to high light shows that the rate of L-Gal synthesis is higher in these plants. The effect persists after a short period (4 h) in low light, and L-Gal does not increase within 4 h of transfer to high light. This strongly suggests that the difference in capacity to synthesize L-Gal is determined by coarse control of enzyme amount, rather than control through substrate availability or regulatory properties of the enzymes. Further studies of the effect of light intensity on gene expression and enzyme activity in the upstream part of the pathway are in progress, and should reveal which steps have the most control over L-Gal synthesis. This will provide important information for developing metabolic engineering strategies to increase ascorbate biosynthesis.

Increased peroxidase activity in low-ascorbate plants suggests a role for ascorbate in controlling hydrogen peroxide concentration

The antisense L-GalDH plants will ultimately provide a tool for understanding the functions of ascorbate. They have an advantage over the low-ascorbate *vtc* mutants (Conklin *et al.*, 1996; Conklin *et al.*, 2000; Smirnov, 2000b; Veljovic-Jovanovic *et al.*, 2001) and the antisense GDP-mannose pyrophosphorylase potatoes (Keller *et al.*, 1999) that have been studied to date because the block is targeted to a step predicted to have minimal effects on other aspects of carbohydrate metabolism. Indeed, a mutation that completely removes GDP-Man pyrophosphorylase activity (*cyt1*) is embryo-lethal (Lukowitz *et al.*, 2001). However, because ascorbate reduction in the antisense plants is light-dependent, so far we have investigated only the effect of short-term acclimation to high light on selected antioxidants, and we have not yet assessed effects on growth. GSH, the other major hydrophilic small molecule antioxidant in plants, showed a tendency to reduced concentration in low-ascorbate lines, although this response has not been observed in the low-ascorbate *vtc1* mutant (Conklin *et al.*, 1996; Veljovic-Jovanovic *et al.*, 2001). α -Tocopherol is a membrane-localized singlet oxygen scavenger and lipid peroxidation inhibitor. Its oxidized radical form is regenerated to α -tocopherol by ascorbate (Davey *et al.*, 2000), but its concentration was not affected in low-ascorbate plants. A number of other enzymes (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase), involved in regenerating ascorbate from its oxidized forms (Noctor and Foyer, 1998), were also unaffected. This is similar to results from *vtc1* (Conklin *et al.*, 1997; Veljovic-Jovanovic *et al.*, 2001). Ascorbate peroxidase (APX) and peroxidase activity measured with pyrogallol as substrate (PPX) both increased in activity in a nearly linear manner as ascorbate content decreased in five independent antisense L-GalDH lines. This exactly parallels

the higher APX and PPX activity found in *vtc1* compared to wild-type *A. thaliana* (Veljovic-Jovanovic *et al.*, 2001) and suggests that increased peroxidase activity is a robust response to ascorbate deficiency. Most of the increase in APX activity in *vtc1* could be attributed to the cytosolic form (Veljovic-Jovanovic *et al.*, 2001). Veljovic-Jovanovic *et al.* (2001) interpreted the increased PPX activity in relation to reduced growth of *vtc1*, and suggested that increased wall polymer cross-linking caused by high PPX activity in the cell wall could limit growth. An alternative explanation is that the increase in cytosolic APX and PPX is a mechanism to control hydrogen peroxide concentration – increased APX activity may compensate for a lower concentration of its substrate ascorbate, while PPX uses other substrates to reduce hydrogen peroxide. More information is needed on the substrates and localization of the PPX activity. Catalase also destroys hydrogen peroxide, but APX and ascorbate have higher affinity and are more widely distributed in the cell, and probably exert fine control (Karpinski *et al.*, 1999). As hydrogen peroxide is important in initiating signals for response to oxidative stress, pathogen attack and ABA-induced stomatal closure (Desikan *et al.*, 2001; Kovtun *et al.*, 2000; Pei *et al.*, 2000), but could cause damage if too concentrated, the induction of peroxidases in low-ascorbate plants may reveal a mechanism that senses and exerts fine control over hydrogen peroxide concentration. Hydrogen peroxide concentration in *vtc1* is the same as in the wild type (Veljovic-Jovanovic *et al.*, 2001), perhaps indicating this control in operation.

Experimental procedures

Plant material

Tobacco (*Nicotiana tabacum* SRI) was grown *in vitro* on MS basal medium (Murashige and Skoog, 1962) containing 3% sucrose at 25°C, 50 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ photon flux density and 16 h photoperiod. For physiological measurements, 10 individuals per line were grown in soil and randomized in a glasshouse with supplementary lighting to provide high light intensity.

Arabidopsis thaliana was grown *in vitro* on half-strength MS basal medium containing 1% sucrose at 20°C, 50 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ photon flux density and 12 h photoperiod. For physiological measurements, 100 individuals per line were grown in soil in controlled environment chambers at 20°C, 12 h photoperiod and photon flux densities from 40 to 400 $\mu\text{mol m}^{-2} \text{sec}^{-1}$.

L-Galactose dehydrogenase activity

Escherichia coli cells were sonicated in 50 mM Tris-HCl (pH 7.5) containing 20% glycerol, 1 mM EDTA and 2 mM dithiothreitol (DTT) and centrifuged at 9000 *g* for 10 min. Plant tissue (0.2 g FW ml^{-1}) was homogenized in 50 mM Tris-HCl (pH 7.5) containing 20% glycerol, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 1% polyvinylpyrrolidone and centrifuged in a microcentrifuge at maximum speed (12 000 *g*) for 5 min. The *E. coli* and pea seedling supernatants were assayed for L-GalDH activity by NADH

formation at 340 nm in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.1 mM NAD and 5 mM L-galactose. Other plant extracts were assayed by a radioactive assay. Supernatant (6 μ l) was incubated with 0.02 μ Ci L-[1-¹⁴C]L-galactose (55 mCi mmol⁻¹, American Radiochemicals, Boston, MA) and 0.4 mM NAD⁺ in a total volume of 20 μ l 25 mM Tris-HCl (pH 7.5) at room temperature. The reaction was stopped after 20 min by adding 20 μ l ethanol. The product, L-[1-¹⁴C]galactono-1,4-lactone, was separated from L-galactose by TLC on silica plates impregnated with 0.3 M sodium dihydrogen orthophosphate and acetone/butanol/water (8 : 1 : 1) as solvent. TLC plates were scanned with a Berthold Linear Analyser.

Purification of pea *L-GalDH*

Epicotyls from 6-day-old dark-grown peas (*Pisum sativum* cv. Meteor) were homogenized in the extraction medium described above. The homogenate was filtered through two layers of muslin and centrifuged at 30 000 *g* for 20 min at 4°C. L-GalDH activity was precipitated by ammonium sulphate and was found in the 50–70% saturation pellet which was resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 2 mM DTT and 20% glycerol. The enzyme was applied to a phenyl sepharose (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) column equilibrated with 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT and 1 M ammonium sulphate, and eluted with the same buffer over a decreasing gradient of ammonium sulphate concentration. The active fractions were pooled and dialysed against 25 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 12 h. Anion exchange chromatography was performed using DEAE-Sephacel gel (Amersham Bioscience) equilibrated with 25 mM Tris-HCl (pH 8.5), 1 mM EDTA and 1 mM DTT. L-GalDH activity was eluted with a gradient of increasing KCl concentration. Active fractions were pooled and dialysed. Affinity chromatography was performed using Cibacron Blue Sepharose (Amersham Bioscience) equilibrated with 25 mM Tris-HCl (pH 7.5). L-GalDH activity was eluted with 10 mM NAD⁺ over a gradient from pH 7.5–8.5. Active fractions were pooled and concentrated osmotically (by immersing the extract in a dialysis tube into solid polyethylene glycol, PEG 8000), followed by a centrifugal concentrator with a 10 kDa pore size (Millipore UK Ltd., Watford, UK). The concentrated extract was loaded onto a Superose 12 HR size exclusion column (Amersham Bioscience) equilibrated with 25 mM Tris-HCl (pH 7.5), 1 mM EDTA and 1 mM DTT, and eluted with the same buffer. The column was calibrated with cytochrome *c* (12.2 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and amylase (200 kDa). During purification, L-GalDH activity was monitored in a microtitre plate assay of 55 μ l total volume, modified from that described above by addition of nitroblue-tetrazolium (0.35 mg ml⁻¹) and phenazine methosulphate (0.07 mg ml⁻¹). Extract (5 μ l) was added to each well, and absorbance at 620 nm was measured after 20 min using an Anthos ht11 microtitre plate reader. Fractions containing GalDH were pooled and the protein sequenced by Edman degradation.

Isolation of mitochondria

Mitochondria were isolated by differential centrifugation (Moore and Proudlove, 1983). Pea roots were homogenized in 30 mM HEPES pH 7.5, 0.1% bovine serum albumin (fatty acid free), 1 mM EDTA, 0.3 M D-mannitol, 0.6% PVP-25 and 3 mM mercaptoethanol. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 1300 *g* at 4°C for 20 min. The supernatant was

centrifuged at 12 000 *g* at 4°C for 20 min to pellet mitochondria. This pellet was suspended in 10 mM KH₂PO₄ (pH 7.4), 0.1% bovine serum albumin (fatty acid-free), 1 mM EDTA and 0.3 M D-mannitol, and centrifuged again. The resulting pellet was used for assays. Fumarase, succinate dehydrogenase (Moore and Proudlove, 1983) and L-galactono-1,4-lactone dehydrogenase (Bartoli *et al.*, 2000) were used as mitochondrial marker enzymes.

Cloning of *Arabidopsis thaliana L-GalDH* cDNA by RT-PCR

Total RNA from *A. thaliana* seedlings was isolated by phenol-chloroform extraction (Sambrook *et al.*, 1989). RNA yield was quantified spectrophotometrically. Reverse transcription was performed with 5 μ g total RNA, 0.5 μ g oligo-dT (T15), 30 U RNase inhibitor (Promega, UK Ltd., Southampton, UK) and 200 U M-MLV reverse transcriptase (Promega) in 25 μ l of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and 0.8 mM dNTPs for 2 h at 37°C after denaturing the RNA for 5 min at 70°C. The purification of the cDNA with the QIAquick PCR Purification Kit (Qiagen Ltd., Crawley, West Sussex, UK) according to the manufacturer's protocol resulted in 50 μ l cDNA solution. Of this solution, 5 μ l were used as template for PCR with 50 pmol of each gene-specific primer ATH1 (5'-TCACACATGACGAAAATA-GAGCTTCG-3') and ATH2 (5'-CTTCTTTAGTTCTGATGGATTC-CACTTG-3') in 50 μ l of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100 and 1 U Taq polymerase (Promega). After denaturation at 94°C for 4 min, amplification was performed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 1.5 min at 72°C, followed by 5 min at 72°C. After electrophoresis in 1% agarose, the 960 bp fragment was excised and purified from the gel with the QIAquick Gel Extraction Kit (Qiagen). The purified DNA was cloned into the p-GEM-T vector (Promega) according to the manufacturer's protocol. The resulting plasmid pGEM-T-LGalDH was purified from *E. coli* DH5 α as described by Sambrook *et al.* (1989). The insert was sequenced in both directions with M13 primers using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer.

Expression of recombinant *L-GalDH* in *E. coli*

The *L-GalDH* cDNA was subcloned into pBluescript (Stratagene, La Jolla, CA, USA). The plasmid pGem-T-LGalDH was digested with *Apal*, overhanging ends filled with Klenow enzyme and then digested with *PstI*. The 960 bp fragment was purified after electrophoresis from a 1% agarose gel and ligated into the *SmaI/PstI*-digested pBluescript. From the resulting plasmid, the *L-GalDH* gene was excised by digestion with *Bam*HI and *Eco*RI, purified from a 1% agarose gel and cloned into the *Bam*HI-*Eco*RI site of the pRSETB expression vector (Invitrogen, Paisley, UK) to produce pRSETB-LGalDH. *Escherichia coli* BL21(DE3)lysS was used as expression host and transformed with the pRSETB-LGalDH plasmid. After induction with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 4 h, the cells were harvested, suspended in 50 mM Tris-HCl (pH 7.5) containing 10% (v/v) glycerol, and sonicated. The cell debris was removed by centrifugation. Recombinant L-GalDH was purified from the supernatant by affinity chromatography using HisBind Resin (Novagen, Madison, WI, USA) according to the manufacturer's protocol. The fractions with L-GalDH activity were additionally purified by passage through a Superdex 200 size-exclusion column (Amersham Biosciences).

Plant transformation

The reporter gene *uidA* of the binary vector pGPTV-KAN (Becker *et al.*, 1992) was replaced by the CaMV 35S promoter. The cDNA encoding the *A. thaliana* L-GalDH was inserted in the *Sma*I site downstream of the CaMV 35S promoter. The insert was prepared by digestion of the plasmid pGem-T-LGalDH with *Sac*II and *Pst*I. Overhanging ends were filled with Klenow enzyme and the L-GalDH fragment purified from a 1% agarose gel. Vector and insert were ligated with T4 DNA ligase according to the manufacturer's instructions, giving a mixture of pGPTV-LGalDH and pGPTV-anti-LGalDH (Figures 3a and 4a). After transformation of *E. coli* DH5 α , the sense and antisense clones were identified by restriction analysis and sequencing. *Agrobacterium tumefaciens* LBA 4404 was transformed with these plasmids as described by Holsters *et al.* (1978). *Nicotiana tabacum* SRI was transformed with pGPTV-LGalDH and pGPTV-35S as control via *Agrobacterium* by the leaf disc method (Horsch *et al.*, 1985). The primary transformants were screened for the presence of the *A. thaliana* L-GalDH gene by PCR. Genomic DNA was isolated with the DNeasy Plant Mini Kit (Qiagen). Using the gene-specific primers GDH1 (5'-AACTTC-TTCGACACCTCCCC-3') and GDH2 (5'-TCATCCCAACCAACACC-GAC-3'), amplification was performed after initial denaturation at 94°C for 4 min by 30 cycles of 0.5 min at 94°C, 0.5 min at 57°C and 1 min at 72°C, followed by 5 min at 72°C. This resulted in a single product of 664 bp for transgenic plants and no products for the wild-type control and false positives. Vector control transformants were identified by PCR with *nptII* specific primers KM1 (5'-ATCTCACCTTGCTCCTGCC-3') and KM2 (5'-CCGCTCAGAAGAA-CTCGTC-3') under identical conditions. Seedlings of the next generation of transgenic tobacco were selected on MS medium containing 100 mg l⁻¹ kanamycin, and these were used for further characterization.

Arabidopsis thaliana was transformed with pGPTV-anti-LGalDH via *Agrobacterium*-mediated transformation of flower buds as described by Clough and Bent (1998). Seedlings were selected on MS agar plates containing 50 mg l⁻¹ kanamycin. PCR with gene-specific primers GDH1 and GDH2, as described for tobacco, also produced a product of 664 bp for positive transformants. The larger 1392 bp PCR product of the endogenous genes including introns was only detected in wild type plants under the given conditions. Seedlings of the next generation of transgenic *A. thaliana* were selected on MS agar plates containing 100 mg l⁻¹ kanamycin, and used for further physiological analysis.

Northern blot analysis

Total RNA was isolated and blotted onto a nylon membrane after electrophoresis, as described by Sambrook *et al.* (1989). A digoxigenin (DIG)-labelled probe was synthesized by PCR with the DIG DNA-labelling mix (Roche Diagnostics Ltd., Lewes, East Sussex, UK) using the primers GDH1 and GDH2 and the plasmid pGEM-T-LGalDH as template. Hybridization and detection were performed as described in the *DIG System User's Guide for Filter Hybridization* (Roche Diagnostics Ltd., Lewes, East Sussex, UK, 1985).

Southern blot analysis

Plant genomic DNA was isolated with the DNeasy Plant Maxi Kit (Qiagen). After restriction digest and electrophoresis, the DNA was blotted on to a nylon membrane after electrophoresis as described by Sambrook *et al.* (1989). Hybridization with a

DIG-labelled probe and detection were performed as described in the *DIG System User's Guide for Filter Hybridization* (Boehringer Mannheim, 1995).

Immunoblot analysis

Proteins were transferred from a 10% SDS-PAGE (Laemmli, 1970) to a polyvinylidene fluoride (PVDF) membrane in a semi-dry blotting apparatus using 0.1 M Tris, 0.192 M glycine, 5% methanol as transfer buffer. Immunodetection of L-GalDH was performed with a polyclonal antibody specific against recombinant L-GalDH (expressed and purified as described; antibodies raised in rabbits by Eurogentec, Seraing, Belgium) and the ECL Western Blotting System (Amersham Biosciences).

L-Galactose content

Leaf tissue (0.3 g FW ml⁻¹) was homogenized in butanol-saturated 1 M formic acid. After centrifugation, the pellets were re-extracted twice and the combined extracts freeze-dried. The residues were dissolved in 0.5 ml 50 mM Tris-HCl (pH 8.5) and filtered through Sep-Pak C18 cartridges (Waters, Elstree, Hertford, UK). For quantitative elution, the cartridges were washed with 5 ml water. The filtrates were freeze-dried again and the residues dissolved in 0.5 ml water. 50 μ l of these samples were incubated with 50 μ l 50 mM Tris-HCl (pH 8.5) containing 0.02% BSA, 0.2 mM NAD⁺ and recombinant L-GalDH for 1 h at room temperature. The reaction was stopped by boiling for 2 min. The formation of NADH was measured by fluorescence ($\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 460$ nm) after adding 1.9 ml 10 mM sodium carbonate (pH 10).

Other analytical methods

Ascorbate and glutathione were extracted from plant tissue in 6% (w/v) trichloroacetic acid. Homogenates were centrifuged for 2 min at 12 000 g. Ascorbate and dehydroascorbate were measured by the method of Kampfenkel *et al.* (1995), scaled down for microtitre plates. Values of ascorbate plus dehydroascorbate are reported because the proportion of dehydroascorbate was constant. Glutathione and oxidized glutathione were measured by the method of Anderson (1985). α -Tocopherol was extracted and measured as described by Fryer *et al.* (1998). Mono-dehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase and ascorbate peroxidase were extracted and measured by the methods described by Smirnoff and Colombé (1988). Peroxidase activity with pyrogallol as substrate was measured in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 20 mM pyrogallol and 10 μ l extract. The reaction was started with 1.5 mM H₂O₂ and pyrogallol oxidation was followed at 430 nm. Protein concentrations were measured as described by Bradford (1976). Error bars on graphs indicate standard deviations calculated from at least three replicate samples.

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EMBL Accession No AJ417563(*Arabidopsis thaliana* L-galactose dehydrogenase gene).