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Feedback Inhibition of Spinach L-Galactose Dehydrogenase by L-Ascorbate

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We have studied the enzymological properties of L-galactose dehydrogenase (L-GalDH), a key enzyme in the biosynthetic pathway of L-ascorbate (AsA) in plants. L-GalDH was purified approximately 560-fold from spinach leaves. The enzyme was a homodimer with a subunit mass of 36 kDa. We also cloned the full-length cDNA of spinach L-GalDH, which contained an open reading frame encoding 322 amino acid residues with a calculated molecular mass of 35,261 Da. The deduced amino acid sequence of the cDNA showed 82, 79 and 75% homology to L-GalDH from kiwifruit, apple and *Arabidopsis*, respectively. Recombinant enzyme expressed from the cDNA in *Escherichia coli* showed L-GalDH activity. Southern blot analysis revealed that the spinach L-*GalDH* gene occurs in a single copy. Northern blot analysis suggests that L-GalDH is expressed in different organs of spinach. The purified native L-GalDH showed high specificity for L-galactose with a K_m of 116.2±3.2 μM. Interestingly, spinach L-GalDH exhibited reversible inhibition by AsA, the end-product of the biosynthetic pathway. The inhibition kinetics indicated a linear-competitive inhibition with a K_i of 133.2±7.2 μM, suggesting feedback regulation in AsA synthesis in the plant.

Keywords: Ascorbate biosynthesis — Feedback inhibition — L-Galactose dehydrogenase — Spinach (*Spinacia oleracea*).

Abbreviations: AOS, active oxygen species; AsA, L-ascorbate; APX, ascorbate peroxidase; DTT, dithiothreitol; LB medium, Luria–Bertani medium; L-Gal, L-galactose; L-GalDH, L-galactose dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; SSC, standard saline citrate.

The nucleotide sequence data reported have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB160990.

Introduction

In photosynthetic organisms, including eukaryotic algae, L-ascorbate (AsA) plays multiple biochemical roles in the antioxidant system (Foyer 1993, Smirnoff 1996, Yoshimura et al. 2000), photosynthesis (Foyer 1993), transmembrane electron transport (Horemans et al. 1994) and cell cycle regulation (Potters et al. 2000). Furthermore, it is involved in crucial physiological processes, such as biosynthesis of the cell wall, phytohormones and secondary metabolites (Smirnoff and Wheeler 2000).

In animals, the biosynthetic pathway of AsA is clearly understood, whereas that of AsA in plants has not been elucidated until relatively recently. In animals, AsA is synthesized in the liver or kidney from D-glucose (D-Glu) through the intermediates D-glucuronate and L-gulono-1,4-lactone (L-GulL) by a mechanism involving inversion of the carbon skeleton (Nishikimi and Yagi 1996). The microsomal L-GulL oxidase oxidizes L-GulL to AsA as the final step of the AsA biosynthesis (Nishikimi et al. 1976).

Recently, Wheeler et al. (1998) proposed a new biosynthetic pathway (the Smirnoff–Wheeler pathway) of AsA in higher plants that involves the conversion of D-Glu to GDP-D-mannose (GDP-D-Man), GDP-L-galactose (GDP-L-Gal), L-galactose (L-Gal), L-galactono-1,4-lactone (L-GalL) and AsA. This biosynthetic pathway is supported by the following facts: (i) an AsA-deficient mutant of *Arabidopsis* (*vtc1*) possesses 30% of the level of wild type AsA and incorporates [^{14}C]D-Glu and [^{14}C]D-Man into AsA more slowly than wild-type (Conklin et al. 1996, Conklin et al. 1997, Conklin et al. 1999), (ii) the *VTC1* gene encodes GDP-D-Man pyrophosphorylase (GMP), which catalyses the conversion from D-Man-1-phosphate to GDP-D-Man (Conklin et al. 1997, Conklin et al. 1999), (iii) antisense suppression of GMP activity in potato plants reduces the level of AsA (Keller et al. 1999), (iv) the conversion of GDP-D-Man to GDP-L-Gal is catalysed by GDP-D-Man-3,5-epimerase (GME), which is partially purified from cell suspension cultures of *Arabidopsis* and for which the corresponding gene was identified (Wolucka et al. 2001), (v) L-Gal dehydrogenase (L-GalDH), which oxidizes L-Gal to L-GalL, has been purified and characterized from pea seedling and *Arabidopsis thaliana* (Wheeler et al. 1998, Gatzek et al. 2002), (vi) antisence suppression of L-GalDH caused a decrease in the AsA pool size under high light irradiation in *Arabidopsis* plants (Gatzek et al. 2002).

Based on recent studies of AsA biosynthesis, much attention has been turned to its regulation. We reported that transcripts of L-GalL dehydrogenase (L-GalLDH), which functions at the last step of the biosynthetic pathway, were expressed in leaves, stems and roots at almost the same level (Yabuta et al. 2000). Furthermore, the transcript levels of L-GalLDH were induced by light along with an increase in the AsA content in

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Table 1 Purification for L-GalDH from spinach

Step	Total protein (mg)	Total activity (μmol h^{-1})	Specific activity [nmol h^{-1} (mg protein)$^{-1}$]	Purification (fold)	Yield (%)
Crude extract	5,760	156.0	27.1	1.0	100
Ultra-centrifugation	4,980	155.4	31.2	1.2	99.6
30–75% $(NH_4)_2SO_2$	3,160	150.4	47.6	1.8	96.4
Q Sepharose	456	112.8	247.4	9.1	72.3
Phenyl Sepharose	36	82.0	2,277.8	84.1	52.6
Superdex 200	0.96	14.5	15,104.2	557.4	9.3

leaves (Tabata et al. 2002, Tamaoki et al. 2003). Recently, Agius et al. (2003) reported that the AsA pool size in ripening strawberry fruit appears to be broadly correlated with the expression level of D-galacturonic acid reductase, which converts D-galacturonic acid into L-galactonic acid, leading finally to the conversion to L-GalL. In addition, overexpression of D-galacturonic acid reductase enhances the AsA content in leaves of *Arabidopsis* (Agius et al. 2003). These data suggest that the AsA pool size may be determined, at least partly, by the level of expression of L-GalLDH or D-galacturonic acid reductase. However, little is known about the molecular machinery that regulates AsA pool size by regulating the enzymes involved in the biosynthetic pathway of AsA.

In the Smirnoff–Wheeler pathway, the dehydrogenation of L-Gal by L-GalDH, an irreversible reaction, can be considered to be the first committed step (Wheeler et al. 1998, Gatzek et al. 2002). Thus, this step could be a point for regulating the flux through the biosynthetic pathway of AsA. The AsA pool size in *Arabidopsis* is higher at high light intensity, but neither L-GalDH activity nor the protein level of L-GalDH is affected in plants that are acclimated to low versus high light.

To explore the regulatory system for the biosynthesis of AsA, we purified from spinach leaves L-GalDH and characterized the cDNA that encodes it. We found competitive inhibition of spinach L-GalDH activity by AsA, suggesting an equilibrium-type of feedback regulation for AsA synthesis.

Results

Purification of L-GalDH from spinach leaves

L-GalDH was purified 557.4-fold with 9.3% recovery from spinach leaves by $(NH_4)_2SO_4$ precipitation, anion exchange chromatography, hydrophobic interaction chromatography and gel filtration (Table 1). The purified enzyme had a specific activity of approximately 15.1±0.3 μmol h^{-1} (mg protein)$^{-1}$. During the purification, the enzyme activity was eluted from all columns as a sharp single peak (data not shown). Furthermore, the enzyme had a molecular mass of 75 kDa as estimated by gel-filtration chromatography. The active fractions from the gel filtration column were further separated by native PAGE, followed by staining for L-GalDH activity. Only a single band was detected with the addition of L-Gal (data not shown). These data indicate that spinach leaves contain only one type of L-GalDH protein.

Properties and kinetic parameters of L-GalDH

We explored the enzymological properties of the L-GalDH purified from spinach leaves. The spinach enzyme utilized L-Gal with the highest specificity, followed by L-gulose (L-Gul) and L-Glu with rates of oxidation of 71.4% and 11.9%, respectively, of that with L-Gal. The enzyme showed no activity with L-fructose, L-Man, L-xylose, L-arabinose, D-Gal, D-Glu, L-fucose, D-Man and D-arabinose. The activity with 1 mM $NADP^+$ was 4.7% of that with 1 mM NAD^+. The spinach L-GalDH obeyed Michaelis–Menten type kinetics toward L-Gal and NAD^+. The apparent K_m values for L-Gal and NAD^+ estimated by Lineweaver–Burk plots were 116.2±3.2 μM and 17.9±0.2 μM, respectively (Fig. 1). These values were comparable with those of L-GalDH from *Arabidopsis*, but the K_m values for L-Gal of these enzymes were lower than that of pea L-GalDH (0.43 mM) (Gatzek et al. 2002). A K_m value for L-Gul of spinach L-GalDH was 1.5±0.1 mM, while the value of pea L-GalDH was 3.7 mM (Gatzek et al. 2002). The optimum pH and temperature of spinach L-GalDH were 9.25 and 40°C, respectively. Previous reports about L-GalDH showed optimum pH ranges of 7.5–9.0 (Wheeler et al. 1998, Gatzek et al. 2002).

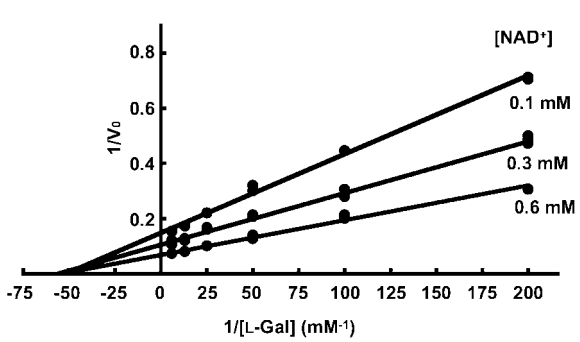

Fig. 1 Affinity of spinach L-GalDH for L-Gal and NAD^+. Initial velocities were assayed as described in Material and Methods with varied concentrations of L-Gal and NAD^+. Reciprocals of relative values of the initial velocities are plotted for each NAD^+ concentration against reciprocals of varying concentrations of L-Gal. The data represent mean values from three repetitive experiments.

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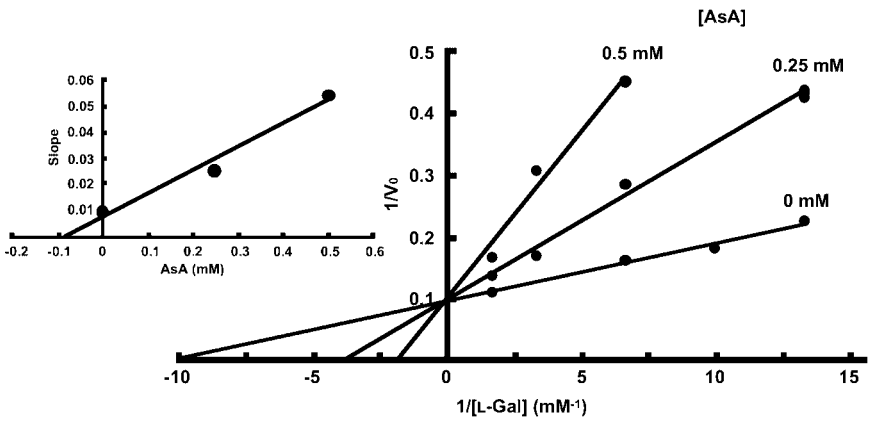

Fig. 2 Inhibition of spinach L-GalDH by AsA. Initial velocities were assayed as described in Material and Methods with AsA at 0, 0.25 or 0.5 mM. The NAD^+ concentration was held constant at 0.1 mM. Reciprocals of relative values of the initial velocities are plotted for each AsA concentration against reciprocals of varying concentrations of L-Gal. The inset figure is the secondary plot of the slopes against AsA concentration. The data represent mean values from three repetitive experiments.

Interestingly, spinach L-GalDH activity was inhibited 41% by 1 mM AsA, the end-product of the biosynthetic pathway. The inhibition kinetics indicated a linear-competitive inhibition with a K_i value of 133.2±7.2 μM (Fig. 2). The extent of inhibition was not changed by prolonged incubation with AsA, and the activity could be restored to the original value by removing the AsA by treatment of the reaction mixture with ascorbate oxidase (Table 2). These data suggest that the inhibition of spinach L-GalDH is reversible, and not due to inactivation of the enzyme. L-GalDH purified from *Arabidopsis* also showed inhibition by AsA (data not shown).

The L-GalDH activity was not affected by the addition of 1 mM dehydro-AsA (DHA) or 1 mM L-GalL. The enzyme activity was stimulated 17% and 28% by 1 mM DTT and 1 mM GSH, respectively, and was inhibited 67% and 92% by 5 mM *N*-ethylmaleimide (NEM) and 0.2 mM *p*-chloromercuribenzoate. However, the activity inhibited by NEM was restored to 71% by incubation with 5 mM GSH. A similar inhibition has been reported for pea L-GalDH (Gatzek et al. 2002). These data indicate that a sulfhydryl group in the enzyme protein participates in the reaction.

Amino acid sequence analysis of L-GalDH peptides

To further purify the enzyme, the active fractions were separated by SDS–PAGE. Silver staining after SDS–PAGE of the partially purified enzyme showed a major band of 36 kDa (data not shown). The protein band was isolated from the gel and used for analysis of the N-terminal amino acid sequence. Unfortunately, we could not determine the N-terminal amino acid sequence due to blockage of the N-terminus. Therefore, in order to determine the amino acid sequences of some internal peptides, the 36 kDa protein was cleaved by digestion with trypsin. Approximately 15 peptides were detected based on absorbance at 210 nm after reverse-phase chromatography, and well-resolved peaks (two peptides) were collected and sequenced. The amino acid sequences of peptides –1 and –2 were as follows: peptide-1: YAEGFDFSAER, peptide-2: GVGVINASPLSM. Both peptides had significant homology to the internal amino acid sequence of L-GalDH from *Arabidopsis* (Gatzek et al. 2002 accession number AJ417563) (Fig. 3).

Isolation and characterization of a cDNA encoding L-GalDH

A 0.4 kbp fragment was amplified with primers P-1S (a degenerate primer coding for peptide-1) and P-2A (a degenerate primer coding for peptide-2). This fragment was cloned into plasmid pSTBlue-1 and the nucleotide sequence was analysed. The fragment encoded a 119-residue polypeptide. Based on the nucleotide sequence obtained, we applied the rapid amplification of cDNA ends (RACE) technique to determine the 5′- and 3′-flanking sequences. The amplified fragments were cloned into pSTBlue-1, and the nucleotide sequences were determined for the approximately 0.4 kbp 5′-extended fragment and 0.6 kbp 3′-extended fragment. A full-length cDNA encoding the L-GalDH protein was amplified using a set of primers

Table 2 Reversibility of spinach L-GalDH inhibition by AsA

L-GalDH activity after incubation	0	15	30 min
Control	100.0 ± 3.0	96.9 ± 3.4	93.2 ± 5.6
0.5 mM AsA	36.4 ± 4.0	31.3 ± 2.9	34.4 ± 3.0
0.5 mM AsA, AOX treatment	98.2 ± 2.0	92.9 ± 1.3	93.9 ± 3.0

Purified spinach L-GalDH was incubated in 100 mM Tris–HCl buffer pH 7.5 with or without (control) 0.5 mM AsA at 37°C. After the indicated times, an aliquot of the incubation mixture was taken out and assayed for L-GalDH activity by adding L-Gal (0.15 mM) and NAD^+ (0.1 mM). One set of samples was treated with ascorbate oxidase (AOX: 5 μmol min^{-1} ml^{-1}) for 5 min before the assay of activity. The initial velocities are presented as percentages relative to those at the start of the incubation. The data represent mean values from three repetitive experiments.

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Fig. 3 Comparison of the deduced amino acid sequences of L-GalDH from spinach, kiwifruit (accession number AY176585), apple (AY264803) and *Arabidopsis* (AJ417563). Gaps were introduced to optimize the alignment. Amino acid residues conserved in more than three sequences are shaded. The asterisks show the consensus amino acid residues in the four species. Boxed residues are N-terminal sequences of products of trypsin digestion determined by protein sequencing.

designed from the sequences of the 5′- or 3′-untranslated region. A 1.2 kbp fragment was amplified, cloned into pSTBlue-1 and sequenced.

The cDNA contained 1,239 bp with an open reading frame of 966 bp encoding 322 amino acids (Fig. 3). The calculated molecular mass of the protein was 35,261.4 Da, which was in agreement with the molecular mass of the purified enzyme. The deduced amino acid sequence contained the sequences obtained experimentally for the two trypsin-digested fragments, suggesting the authenticity of the cloned cDNA. The deduced amino acid sequence of spinach L-GalDH showed 82, 79 and 75% homology to L-GalDH from kiwifruit, apple and *Arabidopsis*, respectively (Fig. 3).

Expression of spinach L-GalDH in Escherichia coli

Next, we expressed the cDNA in a fusion construct with the glutathione *S*-transferase (GST) gene in *E. coli* to obtain recombinant L-GalDH enzyme. The recombinant L-GalDH accounted for nearly 10% of the total protein in the *E. coli* cells, as shown by SDS–PAGE. By one-step purification with a GST-affinity column, we could obtain apparently pure protein that had L-GalDH activity of 2.17±0.07 μmol min^{-1} (mg protein)$^{-1}$. The substrate specificity of the recombinant enzyme was in good agreement with that of the native enzyme (data not shown).

Southern blot analysis

We examined the genomic organization of the gene encoding the L-GalDH protein by Southern blot analysis. When the DNA prepared from spinach leaves was digested with several restriction endonucleases and the fragments were probed with ^{32}P-labelled-spinach L-GalDH cDNA, a single band was detected in both *Eco*RI and *Bam*HI digests (Fig. 4), suggesting that there is a single copy of the *L-GalDH* gene in the spinach

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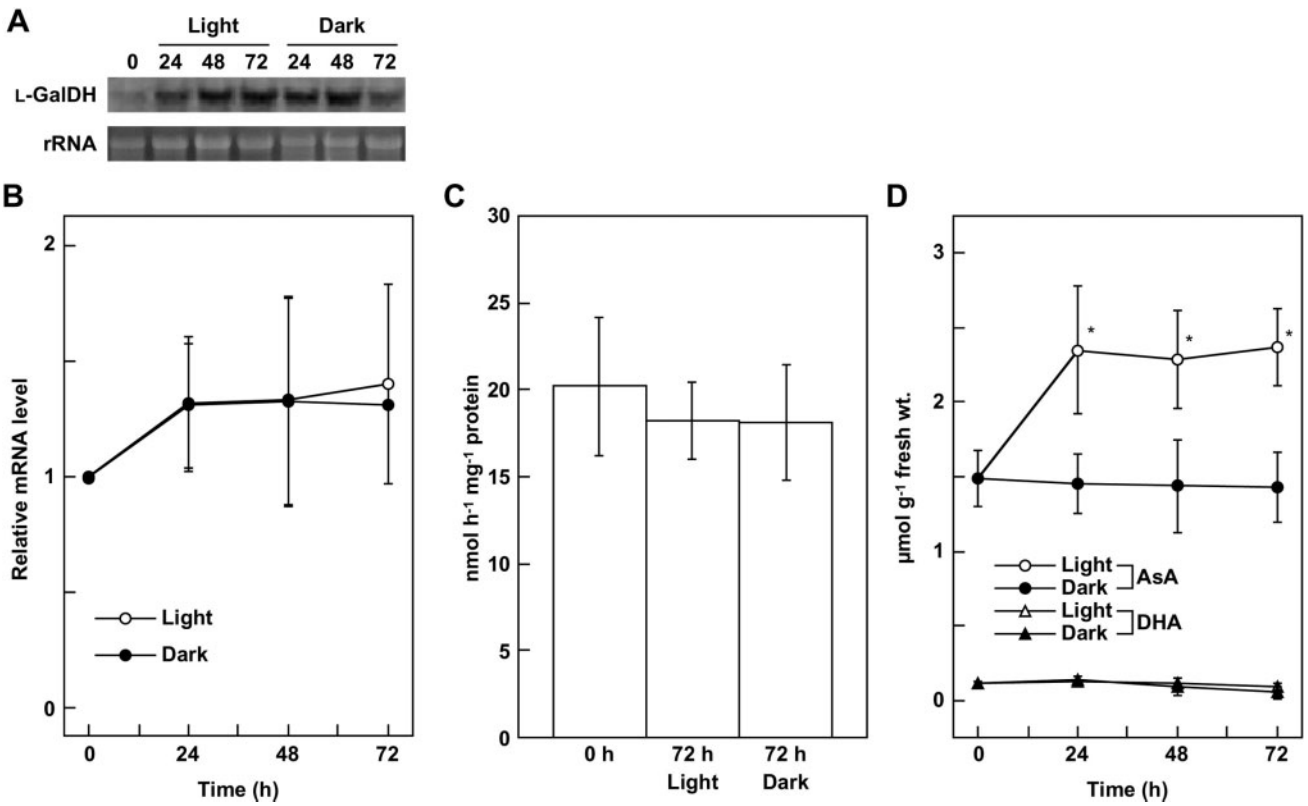

Fig. 6 Changes in AsA/DHA contents, and the transcript and activity levels of L-GalDH in greening spinach cotyledons. (A) Northern blot analysis. See the legend to Fig. 5 for details. (B) Relative mRNA levels. The mRNA levels of each sample were quantified using a Mac BAS 2000, normalized by the respective 18S ribosomal RNA level, and expressed as the mean value ± SD of three individual experiments. The value at time 0 was set to 1. (C) Activities. (D) AsA and DHA contents. The data shown are the mean values ± SD of three individual experiments. Asterisks indicate that the mean values are significantly different compared with those of the control plants ($P < 0.05$).

genome. This result agreed with the elution profile of L-GalDH during purification. Similarly, *L-GalDH* from *Arabidopsis* is encoded by a single copy gene (Gatzek et al. 2002).

Expression of L-GalDH in different organs

To understand the role of L-GalDH in different organs, we estimated the activities and transcript levels of L-GalDH and AsA levels in leaf, stem and root of mature spinach plants (Fig. 5). Comparable levels of both the activity and transcript abundance suggest that L-GalDH plays an active role in AsA biosynthesis in different organs. We also examined the activity and transcript levels of L-GalDH during germination. When 2-week-old dark-grown etiolated seedlings were illuminated at 50 µE m^{-2} s^{-1}, the AsA content increased until 24 h and was then maintained at a constant level. In contrast, the AsA level was not altered in etiolated cotyledons. However, no significant differences in the activity or the steady-state transcript levels of L-GalDH were found between the greening and etiolated cotyledons (Fig. 6).

Discussion

In order to study the enzymological properties of L-GalDH, in this work we purified the enzyme from spinach leaves. So far, L-GalDH has been purified only from pea (Gatzek et al. 2002). The spinach L-GalDH was apparently located in the cytosol because we purified the enzyme from the soluble fraction and the corresponding cDNA encodes no known signal peptide. The enzyme was a homodimer with a subunit mass of 36 kDa, whereas pea L-GalDH was a homotetramer (Gatzek et al. 2002). Compared with pea L-GalDH, spinach L-GalDH showed higher affinity for L-Gal and NAD^+ (Fig. 1).

Because the only known metabolic fate of L-GalL is oxidation into AsA, dehydrogenation of L-Gal by L-GalDH can be considered as the first committed step in the Smirnoff–Wheeler pathway (Wheeler et al. 1998). Furthermore, the reaction by L-GalDH has been reported to be irreversible (Gatzek et al. 2002). Thus, the dehydrogenation of L-Gal could be a point for regulating the flux through the pathway. Our finding that spinach L-GalDH is competitively inhibited by the end-product AsA (Fig. 2), which was also observed with *Arabidopsis* L-

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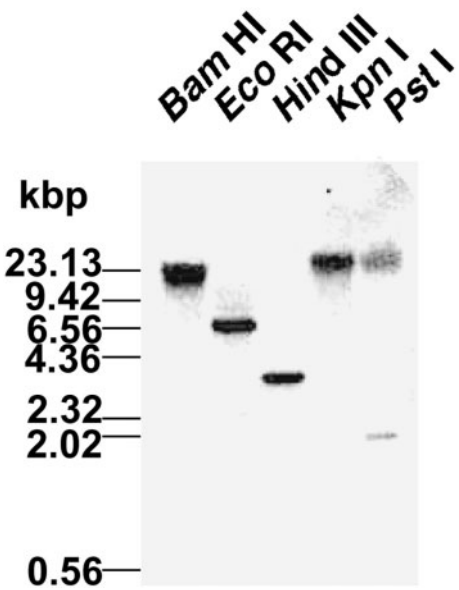

Fig. 4 Southern blot analysis of the L-*GalDH* gene. Genomic DNA (20 μg) isolated from spinach leaves was digested to completion with the indicated restriction enzymes, separated by agarose gel electrophoresis (1% gels) and transferred to a membrane. The gel-blot membrane was hybridized with ^{32}P-labelled full-length cDNA of spinach L-GalDH under high stringency conditions and visualized by autoradiography.

GalDH (data not shown), suggests feedback regulation in AsA biosynthesis in the plant. In fact, feedback control in the AsA biosynthesis has already been demonstrated by Pallanca and Smirnoff (2000). They observed that the rate of AsA synthesis from [U-^{14}C]D-Glu decreased linearly with the increase in the pool size of AsA in embryonic pea seedlings. Given a cytosolic concentration of AsA of as much as 20 mM (Foyer and Lelandais 1996) and the barely detectable levels of L-Gal, the low K_i value of 133 μM for AsA of the spinach L-GalDH means that a large capacity of L-GalDH is under tight control. When barley leaf discs were incubated with L-Gal, AsA content in the leaf tissues increased rapidly several fold (Wheeler et al. 1998). Similarly, when L-GalDH was overexpressed in tobacco plants, there was no increase in the leaf AsA content (Gatzek et al. 2002). Also, antisense suppression of L-GalDH in *Arabidopsis* did not cause a reduction in the AsA pool size under low light intensity (Gatzek et al. 2002). Operation of L-GalDH under feedback inhibition by AsA through K_m modulation may offer an explanation for the above findings.

Recently, Wolucka and Van Montagu (2003) have found that GDP-L-Gul arises as an intermediate during the sequential double epimerization of GDP-D-Man into GDP-L-Gal. They proposed an alternative pathway involving L-GalDH to oxidize L-Gul to L-GulL, and speculated that a cytosolic or a microsomal oxidase/dehydrogenase is responsible for the conversion of L-GulL into AsA. However, even though spinach L-GalDH has a lower K_m of 1.5 mM for L-Gul compared with that of

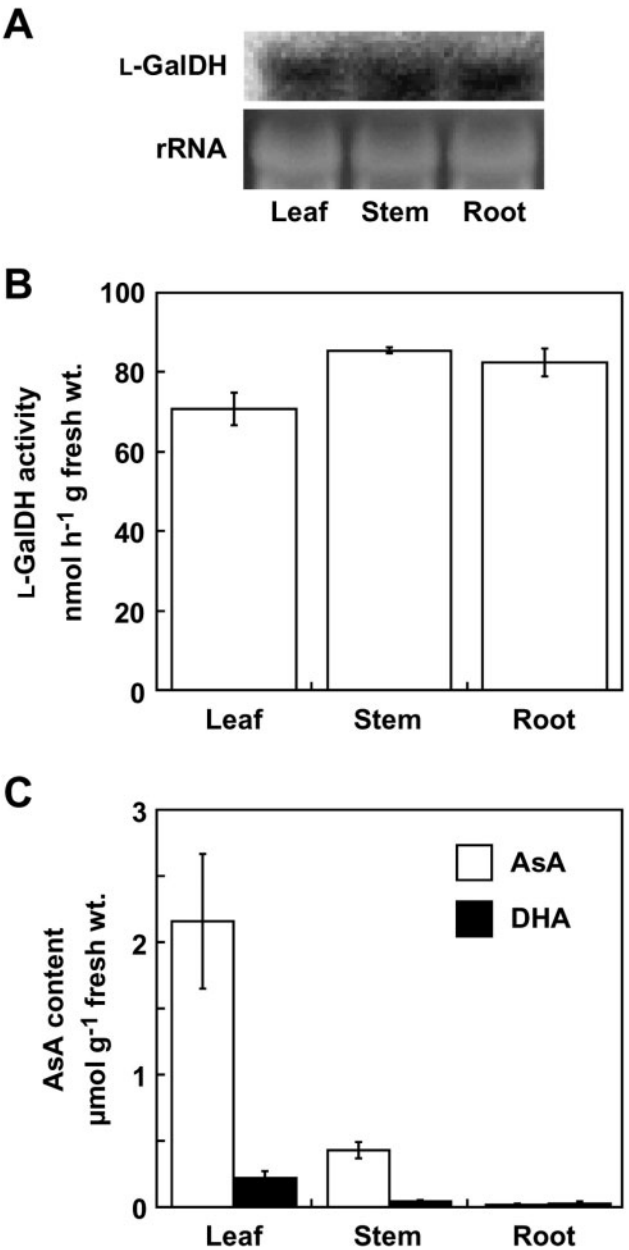

Fig. 5 Activity and transcript levels of L-GalDH, and AsA/DHA contents in different organs of spinach. (A) Northern blot analysis. Total RNA (20 μg) isolated from 7-week-old spinach leaves, stems and roots, was subjected to electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde, and transferred to a membrane. The gel-blot membrane was hybridized with ^{32}P-labelled full-length cDNA of spinach L-GalDH and visualized by autoradiography. Ethidium bromide staining of the rRNA is shown for loading control. The mRNA levels of each sample were quantified using a Mac BAS 2000, normalized by the respective 18S ribosomal RNA level. (B) Activities. Proteins in the soluble fractions were precipitated by $(NH_4)_2SO_4$, dialysed and assayed for L-GalDH activity. (C) AsA and DHA contents. The data shown are the mean values ± SD of three individual experiments.

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3.7 mM in the case of pea L-GalDH, we observed the same degree of inhibition of L-GalDH activity with L-Gul by AsA, as that with L-Gal (data not shown). Accordingly, under the cytosolic concentration of AsA, the apparent K_m of spinach L-GalDH for L-Gul would be several tens of millimolar concentration, which needs to be considered in appreciating the physiological significance of the proposed L-Gul pathway in plants.

The activity and transcript levels of L-GalDH were comparable in different tissues of spinach (Fig. 5), suggesting an active role of L-GalDH in AsA synthesis in different organs. Previously we reported that the transcript levels of mitochondrial L-GalLDH were also comparable in different organs of tobacco (Yabuta et al. 2000). However, as shown in Fig. 5, it has been known that AsA levels vary greatly in different organs (Agius et al. 2003, Tamaoki et al. 2003). Further, AsA levels change in response to different physiological states (Fig. 6). Although our study shows that L-GalDH is competitively inhibited by AsA, the extent of the feedback inhibition in the regulation of AsA in vivo needs to be studied.

Materials and Methods

Materials

Spinach (*Spinacia oleracea*) was purchased from a local market in Nara. L-Gal, L-Gul, L-Glu and L-Man were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All chemicals were of the highest purity grade commercially available.

Enzyme assay

L-GalDH activity was assayed at 37°C in a 3 ml reaction mixture containing 100 mM Tris–HCl pH 7.5, 0.1 mM NAD^+, 0.15 mM L-Gal and the enzyme. The reaction was initiated by the addition of L-Gal. The formation of NADH was measured by fluorescence (λex = 340 nm, λem = 460 nm). The fluorometer was calibrated with freshly prepared NADH standards of 0.3–160 μM in 100 mM Tris–HCl pH 7.5. For specificity of other substrates, L-Gal was replaced by the respective substrate at 1 mM final concentration. Protein was determined by the method described by Bradford (1976).

Partial purification of L-GalDH from spinach leaves

All purification steps were carried out at 4°C. Spinach leaves (2 kg) were homogenized in 4 liter of 100 mM Tris–HCl pH 7.5 containing 20% (v/v) glycerol, 1 mM EDTA and 2 mM dithiothreitol (DTT) (buffer A) using a cooled blender. The homogenate was filtered through eight layers of cloth and then centrifuged at 10,000×*g* for 20 min. The supernatant was subjected to ultra-centrifugation at 100,000×*g* for 30 min. The second supernatant was fractioned with $(NH_4)_2SO_4$, and the pellet precipitated between 30 and 75% saturation was dissolved in 50 ml of buffer A. The enzyme solution was dialysed against 5 liter of buffer A for 12 h, and then was applied to a Q Sepharose HiLoad 16/10 column (FPLC system; Amersham Biosciences, Uppsala, Sweden) equilibrated with buffer A. The column was washed with 100 ml of buffer A and developed with a 136 ml linear gradient of NaCl (0–0.5 M) at a flow rate of 0.8 ml min^{-1}. The activity of L-GalDH was eluted as a single peak at 0.2 M NaCl. $(NH_4)_2SO_4$ at 30% saturation was added to the active fractions. The precipitate was removed by centrifugation at 100,000×*g* for 30 min and then the supernatant was loaded onto a HiLoad 16/10 Phenyl Sepharose column (FPLC system) equilibrated with 30% saturated $(NH_4)_2SO_4$ in buffer A. The enzyme was eluted with a descending gradient of $(NH_4)_2SO_4$ from 30 to 0% saturation in 136 ml of buffer A at an elution rate of 0.8 ml min^{-1}. The active fractions were combined and concentrated by using Centricon-50 (Amicon, Beverly, MA, U.S.A.). The enzyme solution was gel-filtrated with a Superdex 200 HiLoad 16/60 column (FPLC system) equilibrated with solution A including 0.15 M NaCl. To determine the molecular mass of the enzyme, the column was calibrated with molecular markers (molecular weight markers for gel filtration chromatography; Sigma, St. Louis, MO, U.S.A.). The active fractions were combined and stored at –20°C.

Detection of L-GalDH activity in gels

Samples were subjected to discontinuous PAGE under non-denaturing and reducing conditions containing 2 mM DTT according to Laemmli (1970). Electrophoretic separation was performed at 4°C for 2 h with a constant current of 10 mA per gel using 10% (w/v) polyacrylamide gels. The gels were equilibrated with 100 mM Tris–HCl pH 7.5 containing 2 mM DTT at 4°C for a total of 30 min during which the equilibration buffer was changed every 10 min, and then were incubated with 100 mM Tris–HCl pH 7.5 containing 1.5 mM L-Gal, 0.1 mM NAD^+, 0.065 mM phenazine methosulfate and 0.48 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide at 37°C for 30 min.

SDS–PAGE and digestion of L-GalDH

SDS–PAGE was carried out on 12.5% (w/v) polyacrylamide slab gels according to the method of Laemmli (1970). Samples were denatured by boiling for 3 min in 0.1% (w/v) SDS in the presence of 5% (v/v) 2-mercaptoethanol. The gels were transferred to polyvinylidene difluoride (PVDF) membranes using a semidry electroblotting system (model 200/2.0, Bio-Rad, Richmond, CA, U.S.A.) at 13 V for 1 h. The membranes were extensively washed with distilled water, and stained with 0.06% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol for 5 min. The portion of each membrane containing the desired protein band was cut out and destained with 30% aqueous methanol in 7% (v/v) acetic acid for 10 min. The PVDF membrane-bound protein (700 nmol) was digested by trypsin (Sigma) at an enzyme : substrate ratio of 1 : 100 for 15 h at 25°C in 100 mM NH_4HCO_3 pH 7.8, 10% (v/v) acetonitrile and 10 mM $CaCl_2$.

Peptide separation and protein sequencing

The peptides of digested L-GalDH protein were separated by reverse-phase chromatography with a column of μRPC C2/C18 SC 2.1/10 (SMART System, Amersham Biosciences). The gradient elution was done at 0.5 ml min^{-1} with 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 70% 2-propanol : acetonitrile (7 : 3). The separated peptides were transferred to a PVDF membrane using ProSorb™ (Applied Biosystems, Foster City, CA, U.S.A.) and sequenced by automated Edman degradation on a Model 492 pulse-liquid protein sequencer using the standard programming and chemicals provided by Applied Biosystems.

RT–PCR amplification and sequence analysis of a cDNA for L-GalDH

Based on the amino acid sequence obtained, degenerate oligonucleotide primers were designed and used for the amplification of the spinach L-GalDH. Total RNA was prepared from spinach leaves (2.0 g fresh weight) by the procedure of Yoshimura et al. (1999). First-strand cDNA was synthesized using ReverTra Ace (reverse transcriptase; Toyobo, Tokyo, Japan) with an oligo(dT) primer. The reaction was performed in a 20 μl mixture containing a standard enzyme buffer supplied by the manufacturer (Toyobo), 1 μg of total RNA, 0.25 μM oligo(dT) primer, 1 mM dNTPs and 100 units of reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min and then 99°C

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for 5 min. The mixture was used as template cDNA for PCR analysis. PCR amplification was performed as follows: 40 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 60 s and elongation at 72°C for 60 s, followed by incubation at 72°C for 5 min. The reaction mixture (100 µl) contained 1 µM sense primer (P-1S: 5′-TAYGCNGARGGNTTYGAYTT-3′) and 1 µM antisense primer (P-2A: 5′-GCRTTDATNACNCCNACNCC-3′), 200 µM dNTPs, 1.5 units of ExTaq DNA polymerase (Takara Shuzo, Kyoto, Japan) and 10 µl of cDNA. A 0.4 kbp fragment was thereby amplified and then cloned into pSTBlue-1 (Novagen) and sequenced.

Based on the nucleotide sequence obtained, 5′- or 3′-extension of the cDNA clone was performed by the RACE technique using a commercial kit (Gibco-BRL, Rockville, MD, U.S.A.). Amplified fragments were cloned into pSTBlue-1 and the nucleotide sequences of the 5′- and 3′-extended fragments were determined. The following set of primers was designed from the sequences of the 5′- or 3′-non-coding regions to amplify the full-length cDNAs encoding L-GalDH: sense primer, 5′-CCTCCACCGTTACTATTTCCAACTC-3′; antisense primer, 5′-CCAAACAGCCTCTTGAAATTGCTTG-3′. PCR was performed as described above using cDNA from spinach leaves as the template. DNA sequencing was performed by the dideoxy chain primer method using an automatic DNA sequencer (ABI PRISM 310; Applied Biosystems).

Construction of L-GalDH expression plasmid

For the construction of the plasmid to express L-GalDH, the DNA fragment encoding the mature form of spinach L-GalDH was amplified by PCR. The oligonucleotide primers contained an *Eco*RI restriction site (bold sequence), 5′-GAATTCCATGAACACCCATCAAAAATTAGA-3′ and 5′-AGAACACATCTAGTACCTGC-3′. PCR amplification was carried out in a 100 µl reaction mixture containing 10 µl of 10× PCR buffer, 200 µM dNTPs, 2.5 units of Ex*Taq* DNA polymerase, each primer at 1.0 µM and 5 ng of template cDNA. PCR was performed as described above. The DNA fragments generated by PCR were purified by gel electrophoresis and ligated into pSTBlue-1. Following transformation of *E. coli* strain DH5α cells, a clone of the candidate mutant was isolated and sequenced across the region of interest by the dideoxy chain primer method in order to establish the fidelity of L-GalDH construction. From this plasmid digested with *Eco*RI, a 0.9 kbp DNA fragment encoding L-GalDH was isolated, integrated into the pGEX-5X-3 expression vector (Amersham Biosciences) treated with the same restriction enzymes and then introduced into the *E. coli* strain DH5α. The plasmid DNA was prepared from the ampicillin-resistant transformants and the construct was verified by digestion with *Eco*RI. The resulting construct, designated pGX/L-GalDH, was introduced into the *E. coli* strain BL21(DE3)pLysS to test its ability to direct the synthesis of recombinant L-GalDH.

Production of the recombinant enzyme in E. coli

E. coli transformed with pGX/L-GalDH was grown in 50 ml of Luria–Bertani (LB) medium supplemented with ampicillin (50 µg ml^{-1}) at 37°C overnight. The culture was then transferred to 1 liter of LB medium. When the culture reached an absorbance of 0.6 at 600 nm, 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added, and the bacteria were grown for a further 6 h at 37°C. The cells were harvested by centrifugation at 6,000×*g* for 10 min, and the pellets were kept frozen at –20°C.

GST-affinity chromatography of the recombinant L-GalDH

All purification steps were carried out at 4°C. The recombinant *E. coli* cells (3.8 g wet weight) transformed with pGX/L-GalDH were resuspended in 20 ml of 100 mM Tris–HCl pH 7.5 containing 140 mM NaCl (buffer B) and sonicated (10 kHz) using five 10 s strokes at an interval of 30 s. This lysate was centrifuged at 15,000×*g* for 20 min. The supernatant obtained was subjected to ultracentrifugation at 100,000×*g* for 30 min. The supernatant was then loaded onto a 1-ml GSTrap FF column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer B. The column was washed with 10 volumes of buffer B and the recombinant L-GalDH was eluted with 3 vol of buffer B containing 10 mM GSH.

Southern blot analysis

Total DNA was prepared from spinach leaves (20 g fresh wt) by the procedure of Ishikawa et al. (1997). The DNA (20 µg) was digested to completion with various restriction enzymes, separated by agarose gel electrophoresis (1% gel) and transferred to a Hybond N^+ membrane (Amersham Biosciences) using a Model 785 Vacuum Blotter according to the manufacturer's instructions (Bio-Rad). After transfer of the DNA to a membrane, the genomic DNA fragments for L-GalDH were detected by probing with the ^{32}P-random-primed L-GalDH cDNA using a Random Primed DNA Labeling Kit (Takara Shuzo, Kyoto, Japan). The membrane was washed in 1× standard saline citrate (SSC), 0.1× SSC, 0.1% SDS at 68°C. The membrane was then exposed to an imaging plate using Mac BAS 2000 (Fuji Photofilm, Tokyo, Japan).

Northern blot analysis

Total RNA (20 µg each) was subjected to electrophoresis on 1.2% (w/v) agarose gels containing 2.2 M formaldehyde and transferred to a Hybond N^+ membrane. The membrane was prehybridized at 55°C for 3 h in a buffer containing 6× SSC, 5× Denhardt's solution, 1% (w/v) SDS and 100 µg ml^{-1} denatured salmon sperm DNA. The membrane was hybridized at 60°C for 12 h in the presence of ^{32}P-random-primed cDNA of spinach L-GalDH and washed twice at room temperature in 2× SSC, 0.1% SDS for 10 min each, and in 0.1× SSC, 0.1% SDS at 65°C for 1 h. The membrane was then exposed to an imaging plate, and the relative expression level of the L-GalDH transcript was estimated using a Mac BAS 2000 and expressed as the mean value from three individual experiments.

Determination of AsA and DHA

AsA and DHA were measured as described by Yoshimura et al. (2000).

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