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Abstract:	Ascorbic acid (AA) is one of the most powerful natural antioxidant able to prevent enzymatic browning after exogenous treatment of minimally-processed products. The specific mechanism by which AA prevents enzymatic browning remains still debated and a direct effect of endogenous AA stimulation and browning has never been studied. The manipulation of AA pathway is a promising approach to study the biochemical mechanism by which AA acts as an anti-browning agent. In this work, cDNA of L-galactono-1,4-lactone dehydrogenase (GLDH), one of the key gene of the Smirnoff-Wheeler's branch of AA biosynthetic pathway, was isolated from lettuce (Lactuca sativa L. cv 'Iceberg'), a species highly prone to browning. The hypothesis that the overexpression of GLDH translates to AA accumulation and reduces the browning phenomena in lettuce leaves after cutting was tested. Our results indicate that transgenic lettuce plants, showing about 19-fold overexpression of GLDH as compared to wild type (WT), had about +30 % of AA concentration in mature leaves. Transgenic plants exhibited reduced browning over the leaves, even after 10 d after cutting, as demonstrated by higher values of L* and lower values of a* than control plants. Overall, these findings provide a first evidence of the role of endogenous AA as browning-preventing agent. The obtainment of T2 transgenic lettuce plants is a promising first step for further investigation addressed to determine the specific mechanism by which AA act as an anti-browning preservative.

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Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browning in leaves of *Lactuca sativa* after cutting

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Abstract

Ascorbic acid (AA) is one of the most powerful natural antioxidant able to prevent enzymatic browning after exogenous treatment of minimally-processed products. The specific mechanism by which AA prevents enzymatic browning remains still debated and a direct effect of endogenous AA stimulation and browning has never been studied. The manipulation of AA pathway is a promising approach to study the biochemical mechanism by which AA acts as an anti-browning agent. In this work, cDNA of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*), one of the key gene of the Smirnoff-Wheeler's branch of AA biosynthetic pathway, was isolated from lettuce (*Lactuca sativa* L. cv 'Iceberg'), a species highly prone to browning. The hypothesis that the overexpression of *GLDH* translates to AA accumulation and reduces the browning phenomena in lettuce leaves after cutting was tested. Our results indicate that transgenic lettuce plants, showing about 19-fold overexpression of *GLDH* as compared to wild type (WT), had about +30 % of AA concentration in mature leaves. Transgenic plants exhibited reduced browning over the leaves, even after 10 d after cutting, as demonstrated by higher values of L* and lower values of *a** than control plants. Overall, these findings provide a first evidence of the role of endogenous AA as browning-preventing agent. The obtainment of T2 transgenic lettuce plants is a promising first step for further investigation addressed to determine the specific mechanism by which AA act as an anti-browning preservative.

Keywords *Agrobacterium*-mediated transformation, *Lactuca sativa*, Ascorbic acid, *L-galactono-1,4-lactone dehydrogenase* gene

Abbreviations

AA	L-Ascorbic acid (sum of reduced and oxidized form of ascorbic acid)
ALO	D-arabinono-γ-lactone oxidase
AsA	Reduced form of ascorbic acid
BAP	6-Benzylaminopurine

DHA	Dehydroascorbate
DTT	Dithiothreitol
ESTs	Expressed Sequence Tags
GDP	Guanosine diphosphate
GGT	GDP-L-galactose guanyltransferase
GLDH	L-galactono-1,4-lactone dehydrogenase
GLO	L-gulono-1,4-lactone oxidase
GUO	D-gluconolactone oxidase
LsGLDH	*Lactuca sativa* L-galactono-1,4-lactone dehydrogenase
MS	Murashige and Skoog medium
NAA	α-Naphthalene-acetic-acid
POXs	Peroxidases
PPOs	Polyphenol oxidases
RACE	Rapid Amplification of cDNA Ends

Introduction

L-Ascorbic acid (AA), commonly known as vitamin C, is essential for human health and its biosynthetic pathway has been elucidated in animal since 1950s (Ishikawa et al. 2006). Despite the key role that AA plays also in plants, especially under stress condition (Gallie 2013), it is only in the last fifteen years that an understanding of its biosynthesis in plants has emerged (Smirnoff 1996).

Among other stressors, the effect of wounding, occurring for example during preparation of minimally-processed produce (or similarly to that induced by pathogens), consist in loss of sub-cellular compartmentalization and release of phenols, which are normally located in cell vacuole. After mechanical cutting, phenols release represents a deleterious effect as those compounds become a high-affinity substrate for browning-related enzymes, such as peroxidases (POXs) or polyphenol oxidases (PPOs) (Saltveit 2000; Degl'Innocenti et al. 2007). This reaction severely compromised the shelf-life of browning-susceptible produce, such as lettuce.

Exogenous AA is commonly utilized as an anti-browning agent, despite its intimal mechanism(s) of action has never yet been clarified. Three main mechanisms have been proposed: (i) AA may act as antioxidant, promoting the regeneration of *O*-quinones and preserving them from polymerization into brown pigments (Walker 1995; Alscher et al. 1997); (ii) AA can bind to histidine residues of PPOs catalytic site, increasing the enzymatic K_m of PPO and reducing the turnover of PPO-triggered oxidized phenols (Osuga et al. 1994); (iii) as a weak acid, AA accumulation may lower cytosolic pH, thus down-regulating the activity of browning-promoting enzymes (POXs and PPOs) after cutting (Vamos-Vigyazo 1981; Landi et al. 2013). Among these three possible hypotheses, the latter appears less probable in lettuce as both PPOs and POXs maintain high activity under a wide range of pH (Landi et al. 2013).

It has been found that leaf vegetables with constitutive high level of AA (such as rocket salad and spinach) result less prone to browning phenomena than do low-containing leaf vegetables, such

as lettuce (Degl'Innocenti et al. 2007, Bottino et al. 2009). Thus, the attempt to increase AA in a browning-sensitive commodity represents a promising first step to elucidate the involvement and mechanism by which AA can mitigate the effect of browning after cutting. In addition, despite in some countries (especially in Europe) transgenic plants are not allow as human and/or animal food source, outside these countries private companies may nevertheless be interested in the possibility of producing ascorbate-enhanced plants for niche markets (Ishikawa et al. 2006).

Despite many works have demonstrated that AA is synthetized from hexose sugars in plants, some steps of AA pathway still remain uncertain. It seems established that AA can be synthetized following three alternative pathways: (i) the myo-inisitol pathway; (ii) the galacturonate pathway, and (iii) the Smirnoff-Wheeler's pathway which involves the generation of AA from L-galactose (Wheeler et al. 1998) (Fig. 1). L-galactose is generated from mannose-1-phosphate by the conversion of guanosine diphosphate (GDP)-mannose to GDP-L-galactose by GDP-mannose-3′,5′-epimerase (Wolucka et al. 2001) which is then converted to L-galactose. The enzyme that catalyses the latter step remained partially missing although GDP-L-galactose guanyltransferase (GGT) has been proposed to convert GDP-L-galactose to L-galactose-1-phosphate (Laing et al. 2007; Zhou et al. 2012). L-galactono-1,4-lactone is synthesized from the oxidation of L-galactose by the NADH-dependent L-galactose dehydrogenase. Finally, L-galactono-1,4-lactone is oxidized to AsA by L-galactono-1,4-lactone dehydrogenase (GLDH) (EC 1.3.2.3).

Many genes involved in AA biosynthesis and recycling have been cloned, and transgenic plants containing modified levels of AA have been generated (reviewed in Hancock and Viola 2002; Zhang et al. 2007; Cruz-Rus et al. 2012). Silencing/overexpression of genes encoding various enzymes in the AA biosynthesis and metabolic network lead to a decrease/increase in AA content (Alhagdow et al. 2007; Pineau et al. 2008; Badejo et al. 2009; Hemavathi-Upadhyaya et al. 2010; Yu et al. 2010; Bulley et al. 2012; Liu et al. 2013). A supported relationship between GLDH activity and AA biosynthesis (Ôba et al. 1994; Wheeler et al. 1998; Tabata et al. 2001; 2002; Tamaoki et al. 2003) has led to suggestion that this step may be a suitable target for manipulation of

AA biosynthesis in plants (Hancock and Viola 2005). Although no clear relationships among the AA content and GLDH protein amount, have been observed in wheat (Bartoli et al. 2005), tobacco (Imai et al. 2009), and tomato (Alhagdow et al. 2007), the overexpression of tobacco *GLDH* in BY-2 cells under the constitutive CAMV35S promoter resulted in up to 4-fold increased enzyme activity and a 60 % increase in the AA pool size (Tokunaga et al. 2005). In addition, antisense suppression of *GLDH* mRNA led to a significant decline in both GLDH activity and AA levels (-30 %) in the transgenic tobacco BY-2 cells (Tabata et al. 2001).

In this study we tested the hypothesis that lettuce (*L. sativa* L. cv 'Iceberg') transgenic plants, which overexpressed a *GLDH* cDNA (*LsGLDH*), translate to increased AA concentration, and consequently less browning appearance in leaves. This work represents the first report in which the AA content has been manipulated by overexpression of *GLDH* cDNA in Compositae, a large family which numbers several edible crops and the first clear evidence that endogenous AA can act as anti-browning compound.

Materials and methods

Plant material and growth condition

L. sativa seeds (cv 'Iceberg', purchased from Blumen, Milan, Italy) were germinated in Petri dishes, on filter papers moistened with distilled water at 23 ± 1 °C in the dark. After three-four days, germinated seeds were transferred to 8 cm diameter pots containing a 60:40 mixture of soil and sand, respectively. Seedlings were grown in a growth chamber at 23 ± 1 °C under a 16-h photoperiod. Irradiances at the top of the seedlings were 500 μmol photons m^{-2} s^{-1} provided by High Pressure Sodium Lamps HPST 400W/E40/H0 (Venture Lighting Italia S.r.l., Milan, Italy).

Isolation of complete *GLDH* mRNA in lettuce

Two expressed sequence tags (ESTs) of *L. sativa* (DY974309) and *L. serriola* (BQ987137) from the TIGR Plant Transcript Assembly (http://blast.jcvi.org/euk-blast/plantta_blast.cgi) and corresponding to fragments of GLDH-related sequences were identified. The EST sequences were used to choose the primers (LAC1F and LAC4R) for PCR amplification of the *GLDH* cDNA in lettuce (Online Resource 1). The primer LAC1F is placed 36 bp before the putative start codon of the *GLDH* gene.

Total RNA was extracted from young leaf blades of 20-day-old lettuce plants with the TriPure Isolation Reagent, according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Total RNA (4 μg), was used with the Superscript™ II pre-amplification kit (Invitrogen S.R.L., Life Technologies, Carlsbad, CA), to produce the first strand cDNA in conditions recommended by manufacturer. One cDNA fragment was obtained with the primer combination LAC1F-LAC4R. The following PCR conditions were used: 94 °C for 4 min, 35 cycles (30 s at 94 °C, 30 s at 64 °C, 60 s at 72 °C), 72 °C for 7 min. The cDNA fragment sequencing allowed choosing primers to use in the 3'RACE (Rapid Amplification of cDNA Ends) approach, according to the manufacturer's instructions (Invitrogen). A 3'RACE was conducted using the *GLDH*-specific primer LAC5F and the Universal Amplification Primer (UAP) 9 (Online Resource 1) with the following PCR conditions: 94 °C for 4 min, 35 cycles (30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C), 72 °C for 7 min.

To obtain a full-length *GLDH* CDS a PCR was performed with the specific primers LAC1F and LAC6R (Online Resource 1). The PCR conditions were: 94 °C for 4 min, 35 cycles (30 s at 94 °C, 30 s at 58 °C, 100 s at 72 °C), 72 °C for 10 min. The PCRs were performed with a Phusion® high-fidelity DNA polymerase (Thermo Scientific, St. Leon, Germany), according to the manufacturer's instructions.

All PCR products were separated using electrophoresis on a 1 % TAE-agarose gel and visualized with Gel Red™ Nucleic Acid Stain (Biotium, Inc. Hayward, CA) under UV light.

Selected amplified products were purified using the Wizard® SV Gel and PCR Clean-UP System (Promega, Madison, WI). Several purified products were inserted into the pGEM®-T easy vector (Promega), and transformed in *Escherichia coli* JM109 competent cells (Promega). Plasmid cDNA was prepared using Wizard® *Plus* Minipreps DNA Purification Kit (Promega). Several clones were automatically sequenced on both strands by MWG Eurofins Operon (Ebersberg, Germany). Sequence data from this article have been deposited in GenBank under the accession number HG810915.2.

Database searches and phylogenetic analysis

Database searches were carried out using the BLAST program at the National Center for Biotechnology Information (NCBI) (Altschul et al. 1997). PROSITE and PFAM databases were searched to identify conserved domains (Bateman et al. 2002; Falquet et al. 2002). Mito ProtII-v1.101 software at the ExPASy Bioinformatics Resource Portal was used for the prediction of putative mitochondrial targeting sequences and cleavage (Claros and Vincens 1996). The deduced GLDH amino acid sequence of lettuce was compared to GLDH sequences of other higher plants. The amino acid sequence from *Volvox carteri* (GenBank accession no. XM002947966) was used as out-group in the phylogenetic analysis. The evolutionary history was inferred using the Minimum Evolution (ME) method (Rzhetsky and Nei 1992). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are expressed as units of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar 2000) at a search level of 1. The Neighbour-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree. All positions containing gaps and missing data

were eliminated from the dataset (Complete deletion option). A total of 543 positions were found in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Semi-quantitative RT-PCR analysis

To analyze *LsGLDH* transcript levels, total RNA extractions were carried out from cotyledons of 5-day-old WT plants (C), internodal stem of 60-day-old plants (ST), young leaf blade (2 cm long) of 20-day-old plants (YL), completely expanded leaf blades of about 30-day-old plants (mature leaf, ML), vegetative shoots (VS) of 20-day-old plants, young inflorescences (YI) of 60-day-old plants. Total RNA was extracted with the TriPure Isolation Reagent, according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). To exclude DNA contamination, digestion of extracts was performed with DNase I-RNase free (Dasit Sciences S.r.l., Cornaredo, Milan, Italy) as previously described (Sambrook and Russell 2001). To determine the integrity of the RNA and to ensure that equal amounts of RNA were added to each reaction, 1 μg of RNA from each sample was separated *via* electrophoresis in formaldehyde-formamide gel.

First strand cDNA was synthesized using iScript™ cDNA synthesis Kit (Bio-Rad) following the manufacturer's instructions. PCRs were performed using gene-specific primers for *LsGLDH* (LsGLDHF and LsGLDHR; Online Resource 1). To normalize the amount of RNA of each sample, an amplification of the constitutively expressed lettuce *Lsβtub3* gene (GenBank accession number AB232706.1), encoding a β-tubulin 3 was carried out using the specific primers Lsβtub3F and Lsβtub3R (Online Resource 1). Primers were designed to amplify a 115, and 96 bp fragments for *LsGLDH* and *Lsβtub3*, respectively. The number of PCR cycles was chosen in the exponential range of amplification. The PCR conditions were 94 °C for 4 min, 28 cycles (30 s at 94 °C, 30 s at 60 °C, 10 s at 72 °C), 5 min at 72 °C for *Lsβtub3*; 94 °C for 4 min, 32 cycles (30 s at 94 °C, 30 s at 59 °C, 10 s at 72 °C), 5 min at 72 °C for *LsGLDH*. Amplifications were carried out with Gene Amp® PCR System 2700 thermocycler (Applied Biosystems). The PCR products were separated by

electrophoresis on a 2.0 % TAE-agarose gel and visualized with Gel Red™ Nucleic Acid Stain under UV light. The experiment was repeated with three independent RNA extractions. The relative amount of each PCR product was quantified using a Bio-Rad Quantity One Software (Bio-Rad Laboratories Inc, Hercules, CA). The relative intensity of each *LsGLDH* product was expressed as percentage with respect to the *Lsβtub3* product (100 %).

Material for the production of transgenic plants

Lettuce seeds (*L. sativa* cv 'Iceberg') were immersed in 70 % ethanol for 1 min and rinsed with sterile distilled water. Later on, seeds were surface sterilized in 10 % (v/v) 'ACE' bleach (Procter & Gamble S.r.l., Rome, Italy) for 15 min under a pressure of 400 mm Hg, followed by three washes in sterile distilled water. The seeds were placed on solidified agar (0.8 % w/v) Murashige and Skoog (MS; 1962) medium with 3 % (w/v) sucrose, at pH 5.7 (20 mL aliquots per 9 cm Petri dish; 10-15 seeds per dish). Seeds were germinated at 23 ± 1 °C (16-h photoperiod, 100 μmol m^{-2} s^{-1}, daylight fluorescent tubes). Cotyledons were excised after 3-5 days for bacterial inoculation.

Construction of *PetE::GLDH* cassette and growth of *Agrobacterium tumefaciens* strain

Forward and reverse primers, LATF and LATR (Table 1) able to insert *Sal*I restriction sites at the end of the *GLDH* cDNA were used. The PCR conditions were: 95 °C for 4 min, 35 cycles (30 s at 94 °C, 30 s at 64 °C, 100 s at 72 °C), 72 °C for 10 min. The PCRs were performed with a Phusion® high-fidelity DNA polymerase (Thermo Scientific), according to the manufacturer's instructions. The PCR products were separated using electrophoresis on a 1 % TAE-agarose gel and visualized with Gel Red™ Nucleic Acid Stain under UV light. The selected amplified product was purified as stated above, inserted into the pGEM®-T easy vector (Promega), and transformed in *Escherichia*

243 *coli* JM109 competent cells (Promega). Plasmid cDNA was prepared as above and both strands of
244 several clones were automatically sequenced.

245 The transcript was then ligated in a pBIN19 derivative binary vector pVDH282 (Frugis et al.,
246 2001) containing an expression cassette (pea plastocyanin promoter *PetE*-NOS terminator). The
247 cDNA was inserted exploiting a *Sal*I restriction site downstream of p*PetE* and upstream of tNOS
248 (Online Resource 2). The resulting binary vector was named pBINGLDH.

249 The pBINGLDH construct was inserted in LBA4404 *A. tumefaciens* strain. Bacteria were
250 grown from -70 °C glycerol stocks at 28 °C on Luria broth (LB) (Sambrook and Russell 2001)
251 semi-solidified with 1.5 % (w/v) Bactoagar (Oxoid) and supplemented with kanamycin sulphate
252 (100 mg L^{-1}) and rifampicin (50 mg L^{-1}). Overnight liquid cultures were incubated at 28 °C on a
253 horizontal rotary shaker (180 rpm) and were initiated by inoculating 20 mL of liquid LB medium,
254 containing kanamycin sulphate (50 mg L^{-1}) and rifampicin (40 mg L^{-1}), into 100 cm^3 conical flasks.
255 Bacterial cultures were grown to an $O.D._{600}$ of 1.0-1.5 prior to inoculation of explants.

256

257 Plant transformation

258

259 Cotyledons excised from 3-5-day-old seedlings were inoculated with *A. tumefaciens* and transgenic
260 shoots regenerated using a modified procedure described by Curtis et al. (1994). Briefly, a total of
261 600 explants were inoculated with *A. tumefaciens* and cultured on the shoot regeneration medium
262 (15-20 explants per Petri dish). Shoot regeneration medium was made up of MS basal medium
263 supplemented with 30 g L^{-1} of sucrose, 0.1 mg L^{-1} of α-naphthalene-acetic-acid (NAA), 0.5 mg L^{-1}
264 6-benzylaminopurine (BAP), 500 mg L^{-1} carbenicillin, 100 mg L^{-1} cefotaxime (Claforam, Roussel
265 Laboratories, Uxbridge, UK), 50 mg L^{-1} kanamycin sulphate and 0.8 % (w/v) Bactoagar (Oxoid,
266 Basingstoke, UK), pH 5.7. Regenerated shoots were rooted *in vitro* before being transferred to the
267 growth chamber under described above conditions. The root induction medium containing MS basal
268 medium supplemented with 30 g L^{-1} of sucrose, 50 mg L^{-1} kanamycin sulphate and 0.8 % (w/v)

Bactoagar, pH 5.7. Twelve independent T0 *PetE::GLDH* plants were selected by a PCR analysis using the primers PCPF and LAC4R (Online Resource 1). The Primer PCPF is placed 89 bp before the 3'end of the *PetE* promoter (GenBank accession number X68313). The PCR conditions were: 95 °C for 4 min, 30 cycles (30 s at 94 °C, 30 s at 63 °C, 60 s at 72 °C), 72 °C for 7 min. The T0 plants were grown until anthesis and self-pollinated to set T1 seeds. Selection for transgenic seedlings (T1 generation) was performed by germinating seeds on MS basal medium supplemented with 200 mg L^{-1} kanamycin sulphate, 30g L^{-1} of sucrose and 0.8 % (w/v) Bactoagar, pH 5.7 and PCR analysis. Rooted green seedlings were grown in growth chamber until anthesis and self-pollinated to set T2 seeds. T2 homozygous seeds were selected as described for selection of transgenic plants (kanamycin-supplemented medium and PCR analysis) and were used for the further characterization of *PetE::GLDH* phenotype.

Gene expression analysis by real-time RT-PCR (qPCR)

Total RNA was extracted from leaf blades of about 30-day-old of both wild type (WT) and T2 homozygous lettuce transgenic plants (*PetE::GLDH*) with the TriPure Isolation Reagent, according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). The total RNA were isolated from sample collected immediately (t_0) or 24 h (t_1), 48 h (t_2) and 72 h (t_3) after the cutting. The RNA integrity was checked by gel electrophoresis and quantified with a microdrop and treated with RQ1 RNase-Free DNase (Promega) following the manufacturer's instructions. First strand cDNA was synthesized using iScript™cDNA synthesis Kit (Bio-Rad) following the manufacturer's instructions. Real-time quantitative RT-PCR (qPCR) was performed using an ABI Prism 7,300 sequence detection system (Applied Biosystems) and gene-specific primers for *LsGLDH* (LsGLDHF and LsGLDHR) and *Lsβtub3* (Lsβtub3F and Lsβtub3R; Online Resource 1). Quantitative PCR was performed using 50 ng of cDNA and iQSYBRGreen Supermix (Bio-Rad Laboratories), according to the manufacturer's instructions. The thermal cycling conditions of RT-

PCR were as follows: stage I 10 s at 50 °C, stage II 3 min at 95 °C, stage III (×40) 5 s at 95 °C + 30 s at 60 °C. Three independent biological replicates were analyzed per each treatment. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Expression values were normalized using the housekeeping gene *Lsβtub3*.

AA determination

AA was spectrophotometrically determined as described by Kampfenkel et al. (1995). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of Fe^{2+} complexed with 2,2'-dipyridyl. AA was determined immediately (t_0) or 24 h (t_1), 48 h (t_2) and 72 h (t_3) after cutting in leaves stored under dark condition at 4 °C in 0.5-L polyethylene terephthalate boxes. Data were expressed as µg AA g^{-1} FW.

Colour determination

Leaf surface colour measurements were carried out at each time after cutting (0, 1, 2, 3, 10 days) in 5 randomly selected leaves. In each selected leaf, colour was monitored in three spots by using standard CIE Lab* colour space coordinates determined by an Ocean Optic HR2000-UV-VIS-NIR spectrometer coupled whit a tungsten halogen DH2000 light source (Ocean Optics, USA). L* represents the lightness of colours (lightness index scale) and ranged from 0 for black to 100 for white; *a** value represents redness and greenness (*a** and –*a**, respectively). After cutting, leaves were stored under dark condition at 4 °C in 0.5-L polyethylene terephthalate boxes for monitoring color changes over time.

Statistical analysis

The experiment was repeated twice with similar results; a representative run is reported herein. Reported data for semi-quantitative RT-PCR, real-time RT-PCR (qPCR), AA content, and colour parameters represent at least the mean ± SD of five biological replications (n = 5 Homogeneity of variance among data was evaluated using Bartlett's test (p = 0.05).). The percentage data were analyzed after arcsine transformation. Means were subjected to two-way analyses of variance (ANOVA) with genotype (G) and storage (S) as variability factor. Mean were separated after Tukey's test (p = 0.05).

Results

Isolation and sequence analysis of *LsGLDH* cDNA

LsGLDH cDNA contains a complete open reading frame (ORF) of 1,833 bp, flanked by 5'- and 3'-untranslated regions (UTR) of 36 and 88 bp, respectively (Online Resource 3). The putative peptide LsGLDH is 610 amino acids long with a theoretical pI of 8.68 and a calculated molecular weight of 69.2 kDa (Fig. 2a). It contains a mitochondrial targeting sequence (probability of export to mitochondria 0.73) with the cleavage site FR/YA similar to other known GLDHs (Fig. 2a). N-terminal sequence analysis of GLDH isolated from cauliflower mitochondria showed that the mature protein starts exactly at the tyrosine (Y) of the predicted cleavage site (Østergaard et al. 1997). It is likely that LsGLDH is processed to a mature protein by a removal of an N-terminal peptide of 107 amino acids, which probably takes place during transport of GLDH into mitochondria. Therefore, the mature LsGLDH protein consists of 503 amino acid residues having a calculated molecular weight of 57.2 kDa and a pI of 6.68. GLDH and related aldonolactone oxidoreductases involved in vitamin C biosynthesis belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family. Members of this family share a two-domain folding topology, comprising a

conserved N-terminal FAD binding domain and a C-terminal cap domain that determines the substrate specificity. The active site is located at the interface of the domains (Fraaije et al. 1998; Leferink et al. 2008). Analysis of LsGLDH amino acid sequence identified a putative N-terminal FAD-binding domain between residues 128 and 264, wherein is located a motif ^{157}VGSGLSP^{163} common to GLDHs characterized from plants (Fig. 2a). From the alignment, it is evident that GLDH in plants lacks the histidine residue involved in covalent flavinylation in GUO, ALO and GLO, but contains a leucine residue instead (Leu161 in LsGLDH, Fig. 2a, Online Resource 3 and Online Resource 4), indicating that the flavin cofactor is non-covalently bound to the protein (Leferink et al. 2008). The essential Glu-Arg pair found in the active site of GLDH from *Arabidopsis thaliana* (Leferink et al. 2009) is also present in LsGLDH (Fig. 2a, Online Resource 3 and Online Resource 4). The arginine 487 is crucial for the stabilization of the anionic form of the reduced FAD cofactor (Leferink et al. 2009); while the glutamic acid 485 is involved in productive substrate binding (Leferink et al. 2009).

Although plant GLDH have been identified as integral membrane proteins of the inner mitochondrial membrane (Siendones et al. 1999; Bartoli et al. 2000; Hancock and Viola 2005), we did not find any transmembrane regions in the sequence of mature LsGLDH. Analogous results was reported for the sequence of *Arabidopsis* GLDH (Leferink et al. 2008).

The domain specific to D-arabinono-1,4-lactone oxidase, which is involved in the final step of the D-erythroascorbic acid biosynthesis pathway, has been also identified (Fig. 2a Online Resource 3).

Sequence and phylogenetic analyses revealed that *LsGLDH* was related with *GLDH* genes of other species (Fig. 2b and Online Resource 5). A search in GenBank database with the BLAST program showed that LsGLDH shared a query coverage of 95-99 % and the highest amino acid identity (73-76 %) with GLDH proteins of *Camellia sinensis* (GenBank accession numbers KF619448), *N. tabacum* (GenBank accession number BAB13368), *Malus domestica* (GenBank

accession number FJ752244), *Fragaria vesca* subsp. *vesca* (GenBank accession number XP_004303609) and *Ipomoea batatas* (GenBank accession number BAA34995; Imai et al. 1998).

Transcription analysis of *LsGLDH* in lettuce organs

The semi-quantitative RT-PCR was used to analyse the steady state levels of *LsGLDH* mRNA in various organs of WT lettuce (Fig. 3a). *LsGLDH* was consistently transcribed in all samples analyzed (i.e., cotyledons, internodal stem, young and mature leaves, vegetative shoots and young inflorescences), and the highest mRNA levels occurred in internodal stem and expanded leaf blades (Fig. 3a, b).

Regeneration of *PetE::GLDH* transgenic plants

Callus production was induced from the proximal end of cotyledon explants infected by *Agrobacterium* on shoot induction medium supplemented with 50 mg L^{-1} kanamycin. Each 15 days, calli were transferred onto fresh shoot induction medium containing 50 mg L^{-1} kanamycin to select transgenic lettuce shoots. After 40-50 days, multiple adventitious shoots from explants infected by *Agrobacterium* were induced (Online Resource 6). Several green shoots were transferred to MS basal medium supplemented with 50 mg L^{-1} kanamycin for rooting (Online Resource 6). The rooted shoots were transplanted into the soil through acclimation steps (see Material and methods section and Online Resource 6). The frequency of independent putatively transgenic plants was low (24/600, 4.0 %). Twelve putative transformed plants (T0) were selected on the basis of the presence of the *PetE::GLDH* construct assessed by a PCR-based method (Fig. 4a). The twelve plants, heterozygous for the transgene *PetE::GLDH*, were grown until anthesis to seed set (Online Resource 6). Several T1 seeds of each progeny germinated on medium supplemented with kanamycin sulphate were grown until maturity for obtaining homozygous T2 seeds. Homozygous

T2 transgenic progenies were also assessed by a PCR-based approach (Fig. 4b and Online Resource 6).

Real-time RT-PCR *GLDH* expression in mature leaves

Former transformation experiment in lettuce with gene under the control of the CaMV 35S promoter failed, resulting in no constitutive expression of the gene of interest (data not shown). Hence, we decided to overexpress the *GDHL* gene under the control of the pea plastocyanin promoter, which is considered a constitutive promoter, although not strong as the CaMV 35S, and slightly light dependent (Pwee and Gray 1993; Brown et al. 2005). Transgenic plants exhibited an increase ($p < 0.05$) in the expression of the gene of almost 19-fold change that proved the successful transformation (Fig. 5a). Moreover, although there was a decrease of the expression levels of *GDHL* upon leaf-cutting, transgenic lettuce showed almost 8-fold change higher levels in comparison to WT.

AA content and colour determination

GLDH overexpressing plants had about +29 % of constitutive (t_0) AA content in mature leaves as compared to WT plants ($p < 0.001$; Fig. 5b). Similar enhancement of AA was maintained after cutting in transgenic lettuce leaves (t_1-t_3 values averaged 127.9 µg g^{-1} FW *versus* 96.6 found in WT; $p < 0.01$).

CIE Lab* colour values highlight as *GLDH* overexpressing plants showed a less steep decline of luminosity after cutting ($p < 0.001$) until to the last day of storage as compared to the WT counterpart (L*, Fig. 5c). From 72 h after cutting, values of greenness increased less steeply in transgenic plants (*a**; Fig. 5d) and remained lower till to the last days of the experiment (10 d after cutting; $p < 0.01$).

Discussion

The manipulation of AA biosynthesis in plants can be a useful strategy to add new insight either in plant basic or applicative research, such instance that oriented toward reduction of loss of minimally-processed produce due to browning phenomena. Obtained results offer the evidence that transformation of lettuce with *GLDH* cDNA effectively lead to: (i) overexpression of *LsGLDH*, even 24, 48 and 72 h after cutting, (ii) incremented concentration of AA (about +30 %) accumulated in mature leaves and (iii) reduced browning phenomena after cutting. In addition, as LsGLDH proteins have never been isolated in a member of Compositae, and thus an in-depth polypeptide investigation and phylogenetic analyses among other plant families are provided here.

The putative LsGLDH polypeptide sequence presented a mitochondrial targeting signal in the amino terminal end, rich in Ala, Leu, Arg, and Ser residues (6, 13, 7, and 18, respectively) and with relatively few Asp, Glu, Ile, and Val residues (0, 3, 3, and 2, respectively). This composition is similar to that reported for other known GLDHs and matched the characteristics of mitochondrial target peptides (von Heije 1986; Pateraki et al. 2004). The existence of a FAD binding domain suggests that the flavin group is involved in the reaction catalysed by GLDH (Ôba et al. 1995). Many aldonolactone oxidoreductases contain a covalently bound FAD cofactor (Salusjärvi et al. 2004; Logan et al. 2007). LsGLDH lacks the histidine involved in covalent attachment of the FAD cofactor, but contains a leucine (Leu161) at this position. Replacement of Leu into His in *Arabidopsis* GLDH revealed that the presence of a histidine at this position does not initiate covalent binding of the cofactor (Leferink et al. 2008). Covalent coupling of the FAD cofactor is likely an autocatalytic process, requiring a preorganized binding site (Fraaije et al. 2000). Based on the analysis of predicted amino acid sequence LsGLDH showed high homology to GLDH proteins of *C. sinensis* and other GLDHs sequences (e.g., *Malus domestica*, *Ipomea batatas* and *Nicotiana tabacum*). Further phylogenetic analysis showed that LsGLDH cluster in the same sub-group of the

449 *Camelia sinensis* GLDH within a major monophyletic clade of the eudicot asterids and separated
450 from members of the rosid clade.

451 Although it has been established that GLDH catalyses the last step of AA biosynthetic
452 pathway in several plant species (Østergaard et al. 1997; Ioannidi et al. 2009; Li et al. 2010; Cocetta
453 et al. 2012; Xu et al. 2013), no clear correlation between AA content and expression of GLDH has
454 always been detected. An extensive analysis performed in cabbage showed that the expression
455 pattern of the major genes in the D-Man/L-Gal branch of AA pathway, including *GLDH*, have a
456 higher expression levels in cultivar with higher AA content (Ren et al. 2013). Similarly, during
457 apple fruit formation, a greater transcription and activity of GLDH in young fruit contributed to
458 increase the AA content (Li et al. 2011). Post-transcriptional regulation of *GLDH* has also been
459 proposed in different organs of some species or under stress condition (Bartoli et al. 2005; Loscos et
460 al. 2008). In our work, the highest levels of *GLDH* mRNA were found in internodal stems and
461 mature leaves of WT lettuce. However, in all the other organs the mRNA was consistently
462 transcribed suggesting that in lettuce *GLDH* is not a tissue-specific prerogative. Together, these
463 results suggest the complexity of AA biosynthesis and that the importance of different enzymes is
464 strictly related to plant species, organ, tissue and developmental stage taken into consideration, but
465 anyway it points out *GLDH* as a promising candidate for AA manipulation.

466 Studies carried out with transgenic plants, in which AA pathway has been manipulated trough
467 overexpression/silencing of GLDH, indicate that the role of this gene in AA biosynthesis is
468 controversial, too. Alhagdow et al. (2007) observed that tomato *GLDH* silencing did not exhibit
469 clear changes in AA contents compared with WT plants. By contrast, in *A. thaliana GGT* transgenic
470 lines had the highest AA accumulation with a 2.9-fold increase to the WT, which was followed by
471 *GLDH* (1.8-fold) and *L-galactose-1-phosphate phosphatase* transgenic lines (1.5-fold) (Zhou et al.
472 2012). In our study, the overexpression of *GLDH* in lettuce plants is consistent with higher AA
473 content in agreement with that observed by Tokunaga et al. (2005).

The higher AA concentration found in transformed lettuce plants correlate with reduced browning development over the leaves after wounding. *GLDH* overexpressing plants showed higher levels of luminosity (L*) than WT after wounding. This is indicative of low levels of browning since browning is associated with the oxidation of phenolics and their polymerization into dark-brown pigments (King et al. 1991; Ke and Salveit 1989). In addition, transgenic plants also showed a less marked increase in *a** values. This parameters is considered related to browning, too (Castañer et al. 1999) since increment of this values are mainly associated with the breakdown of chlorophyll and hence to the reduction of leaf greenness. In agreement, Martin-Diana et al. (2005) found that high temperature treatment of lettuce leaves (50 °C) leading to degradation of browning-related enzyme (such as polyphenol oxidase and peroxidase) reduced the browning appearance after cutting and leaves maintained higher L*, lower *a** values as compared to lettuce leaves treated with lower temperatures (25 °C and 4 °C). In addition, it has been demonstrated that exogenous AA spray over lettuce leaves provoke L* to decrease less markedly than unsprayed leaves, and immersion of lettuce leaves in a solution containing 1% AA exhibited a less pronounced increment in *a** values (Rivera et al. 2006). Overall, these results underline a clear correlation between overexpression of GLDH, increment of AA and the reduction of browning appearance over lettuce leaves. These GLDH transgenic plants represent a first promising model which has revealed the role of endogenous AA in the prevention of browning, but further experiments are necessary to clarify the mechanism by which AA influences the enzymatic browning.

In addition, it has been also revealed that GLDH influences other processes besides AA biosynthesis (Alhagdow et al. 2007; Pineau et al. 2008; Schertl et al. 2012). GLDH is localized in the inner mitochondrial membrane (Siendones et al. 1999), and forms part of a 850-kDa complex that represents a minor form of the respiratory NADH dehydrogenase complex (complex I) (Heazlewood et al. 2003). The characterization of an *Arabidopsis* knock-out mutant lacking the gene encoding GLDH was found to have drastically reduced amounts of complex I (Pineau et al. 2008) and the central metabolism of plant mitochondria was significantly changed, too. Therefore,

this transgenic lettuce also represents a useful tool for experiments aimed to study the basis of complex I and the mitochondrial respiration chain *in sensu lato*.

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718

719 **Figure legends**

720

721 **Fig. 1** Three postulated pathways for ascorbic acid biosynthesis in plants. Among them, only the
722 Smirnoff-Wheeler's branch has been confirmed trough studies with transgenic plants and almost all
723 the enzymes have been characterized in some species. The only missing step remains the
724 conversion of GDP-L-galactose to L-galactose-1-phosphate even though a GDP-L-galactose
725 guanyltransferase has been proposed by Dowdle et al. (2007) to catalyze this reaction.

726

727 **Fig. 2** Sequence analysis of L-galactono-1,4-lactone dehydrogenase (GLDH) from *Lactuca sativa*
728 (LsGLDH). **a** Predicted amino acids sequence of LsGLDH. The cleavage site FR/YA, amino acids
729 for mitochondrial targeting, is in red and bold characters. A motif (^{157}VGSGLSP^{163}), common to
730 other GLDHs, is in orange characters. Within this motif the Leu (L)161 residue is boxed. The FAD-
731 binding domain is in bleu and bold characters. The Glu (E)485 and the Asp (R)487 residues are
732 boxed and in bold-brown character. The domain specific to D-arabinono-1,4-lactone oxidase is
733 underlined. **b** Dendrogram between 14 GLDH proteins. The GLDH amino acid sequences and the
734 relative GenBank accession numbers are reported in Online Resource 5. Consensus tree was
735 inferred using the Minimum Evolution (ME) method. The Neighbor-joining algorithm was used to
736 generate the initial tree. The optimal tree with the sum of branch length = 1.82969450 is shown. All
737 positions containing gaps and missing data were eliminated from the dataset (Complete deletion
738 option). A total of 543 positions in the final dataset were found. The *L. sativa* GLDH is underlined
739 and the percentages of replicate trees in which the associated taxa clustered together in the bootstrap
740 test (100 replicates) are shown next to the branches. Phylogenetic analyses were conducted in
741 MEGA4. The GLDH amino acid sequence from *Volvox carteri* (GenBank accession no.
742 XM002947966) was used as outgroup in the phylogenetic analysis

743

Fig. 3 Steady-state level of *LsGLDH* mRNAs in lettuce organs. **a** Representative results of RT-PCR analysis: the experiments were repeated three times with consistent results. RNA was prepared from cotyledons (C), internodal stems (ST), young leaf blades (YL), completely expanded leaf blade (mature leaves, ML), vegetative shoots (VS) and young inflorescences (YI). RT-PCR was carried out using primers described in Online Resource 1. Details of the PCR conditions are provided in Materials and methods section. Transcript accumulation of the *Lsβtub3* gene was used as an internal amplification control. The RT-PCR products were resolved on a TAE 2.0 % agarose gel. M indicates the PhiX 174 DNA *Hae*III Digest DNA ladder. **b** Results from analyses of the RT-PCR transcript accumulations performed with the Bio-Rad's Quantity One software and expressed as percentage of the *Lsβtub3* product (100 %) ± SD. The values denoted with the same letter are not significantly different at the 0.05 probability level according to Tukey's test

Fig. 4 Screening of putatively transformed plants conducted by a PCR approach with the primer combination PCPF and LAC4R. The size (1,176 bp) of expected PCR product is indicated. **a** T0 putatively transformed plants: three plants (lane 1, 2 and 3) showed a clear signal as well the positive control (lane 5, DNA of vector); lane 4, T0 non-transformed plants (escape); lane 6, DNA from non-transformed wild type (WT) *Lactuca sativa* (L); lane 7, sterile distilled water (H_20). M indicates the PhiX 174 DNA *Hae*III Digest DNA ladder. **b** T2 transformed plants. Six randomly chosen plants (from 5.1 to 5.6) from a homozygous progeny selected in a kanamycin-supplemented medium that showed the expected amplified PCR-product. L, DNA from non-transformed WT *L. sativa*; H_20, sterile distilled water. M indicates the 1Kb XL ladder (5 PRIME)

Fig. 5 *GLDH* transcription levels (**a**), and total ascorbate (AA) (**b**) in a WT line (n = 5) and in an overexpressing line (n = 5) of *Lactuca sativa* at different time points: t_0, immediately after cutting;

t_1, 24 h after cutting; t_2, 48 h after cutting; t_3, 72 h after cutting. For *GLDH* transcription level, data were normalized using *β-tubulin 3* (*Lsβtub3*) as housekeeping gene. Luminosity (L*) level (**c**); and greenness (*a**) evaluated in WT (white circles) and transgenic plants (dark circles) at 1,2,3,10 days after cutting (**d**). Means were subjected to two-way analyses of variance (ANOVA) with genotype (G) and storage (S) as variability factor. Mean were separated after Tukey's test ($p = 0.05$).

Author Contribution Statement: ML and MF equally contributed to this study. ML, MF, LG and CP designed the experiments and analysis. AB, performed *Agrobacterium*-mediated transformation and real time RT-PCR experiments. MS performed database searches and phylogenetic analysis. ML, MF, CP wrote the manuscript.

Key message: For the first time a *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) CDS was isolated from a Compositae. Overexpression of *GLDH* correlate with higher ascorbate content and reduced browning in lettuce leaves after cutting.

Figure

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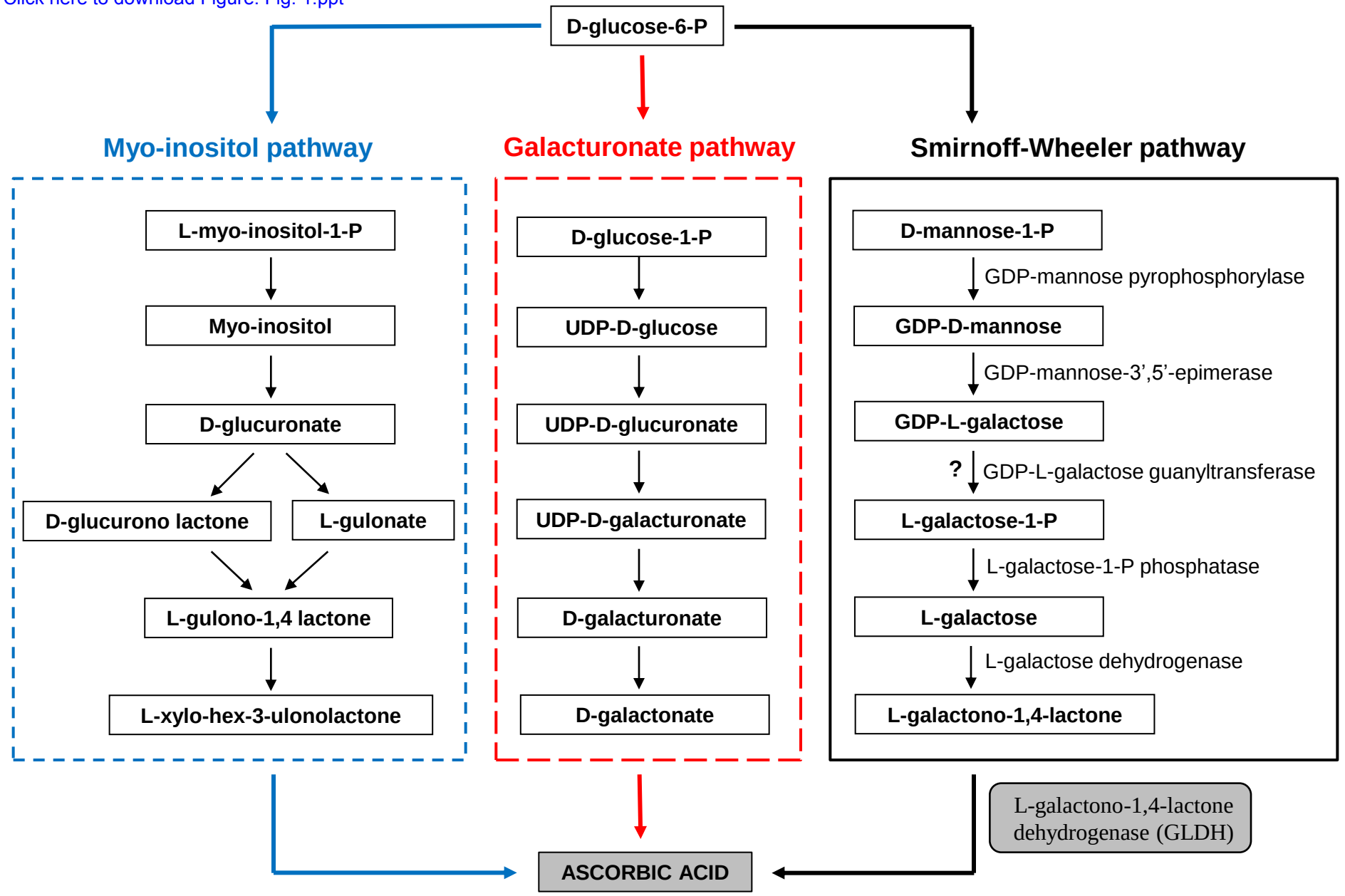

Fig. 1

Figure
Click here to download Figure: Fig. 2.ppt

(a)

```
MLRSLRFQRSLQSSVIHRKNPHFNNTLETLSSSPTTKTPPINLIRQFSSSSPPPPTPPPL 60
SATPSTSSELRKYLGYSALLLSCAVATYYSFPFPENAKHKKAQLFRYAPIPDDLHTVVNW 120
SGTHEVQTRVFLQPESLEELEKIVKDADEKKQKIRPVGSGLSPNGIGLARGGMVNLALMD 180
KVLEVDKEKKTVRVQAGIRVQQLVDVVKDHGITLQNFASIREQQIGGIVQVGAHGTGAKL 240
PPIDEQVISMKLVTPGKGTIEISKDKNPELFYLARCGLGAFGVVAEVTLQCVERQELVEH 300
TFVSNLTEIKKKHKKLLNDNKHVKYLYIPYTDTVVVVTCNPVSKWKGPPKFKPKYSLDEA 360
LQPVRDLYKESLQKYKRQPNENDSKVSDLTFTELRDKLLSIDPLNKDHVKKINEAESEFW 420
KRSEGFRVGWSDEILGFDCGGQQWVSETCFPAGTLSKPNMKDLKFIEEVMELIEKEEIPA 480
PSPIEQRWSASSKSLMSPASSESNDDIFSWVGIIMYLPTSDARQRKQITEEFFHYRHLTQ 540
TRFWNQYSAFEHWAKIEVPKDKNELAALQERLRARFPVDAFNKARKELDPNRILSNAMVE 600
KMFPIEDNAT- 610
```

(b)

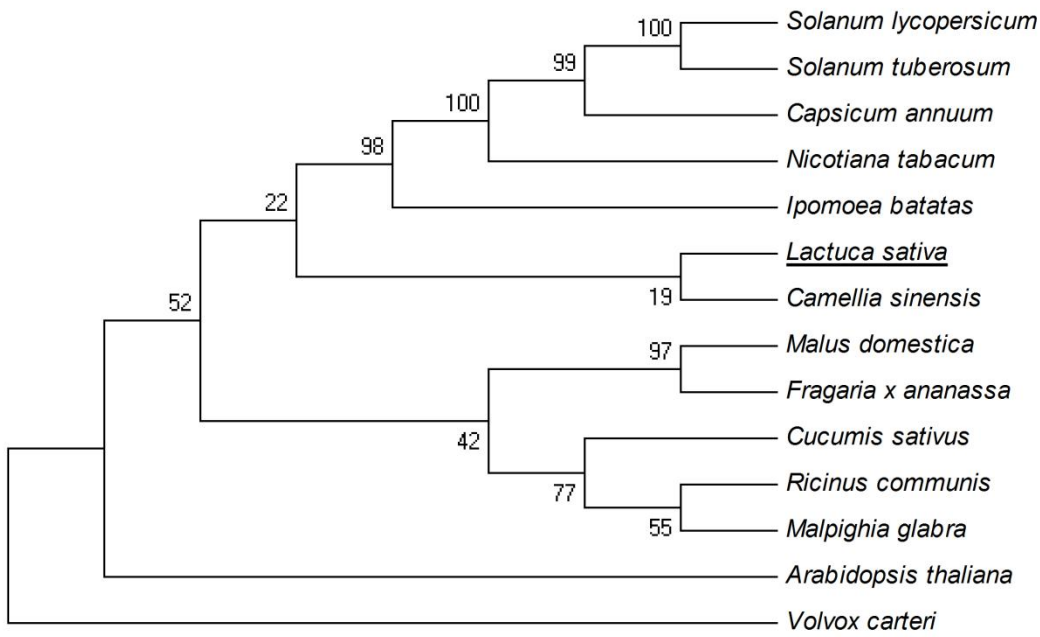

Fig. 2

Figure
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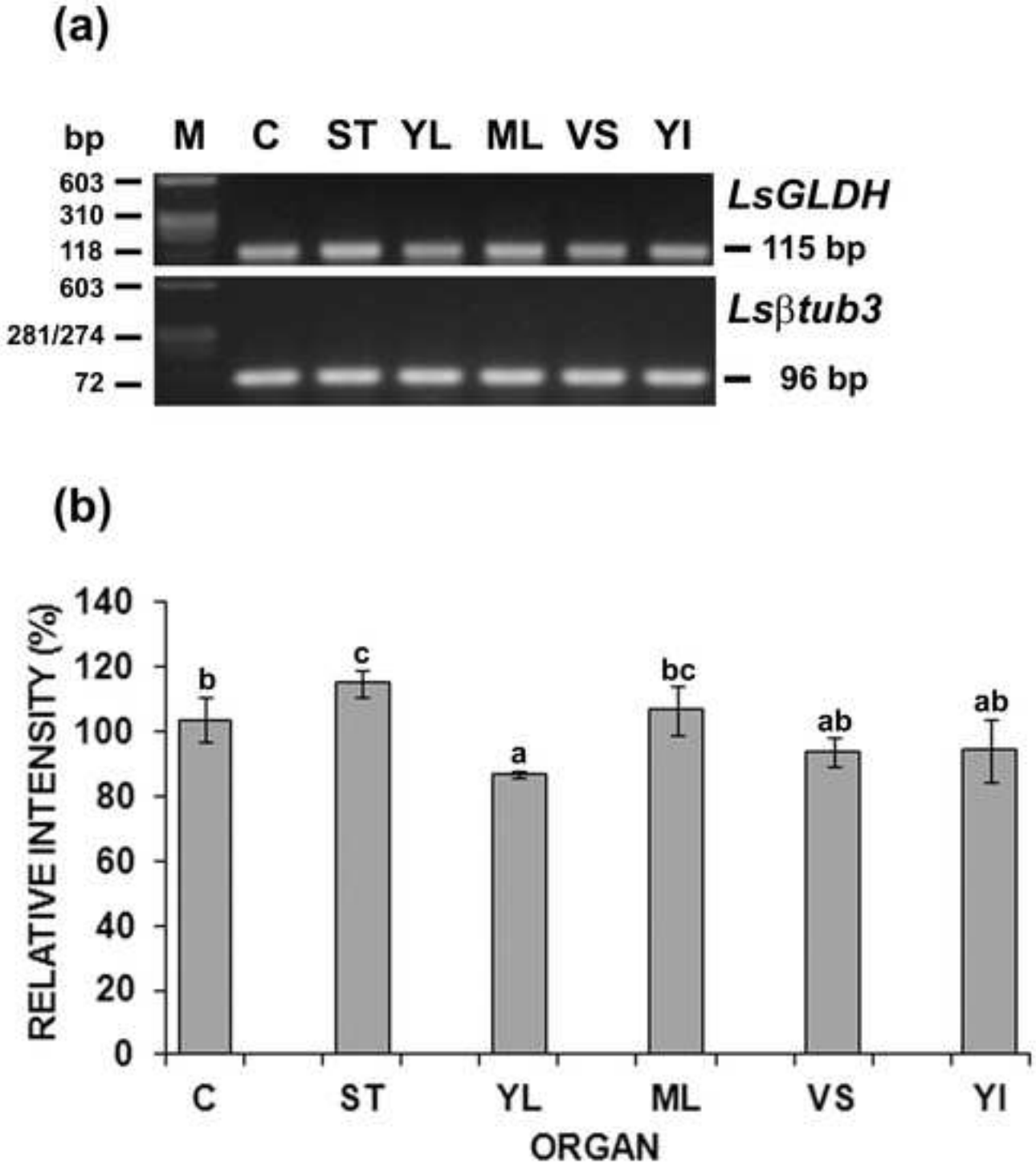

Fig. 3

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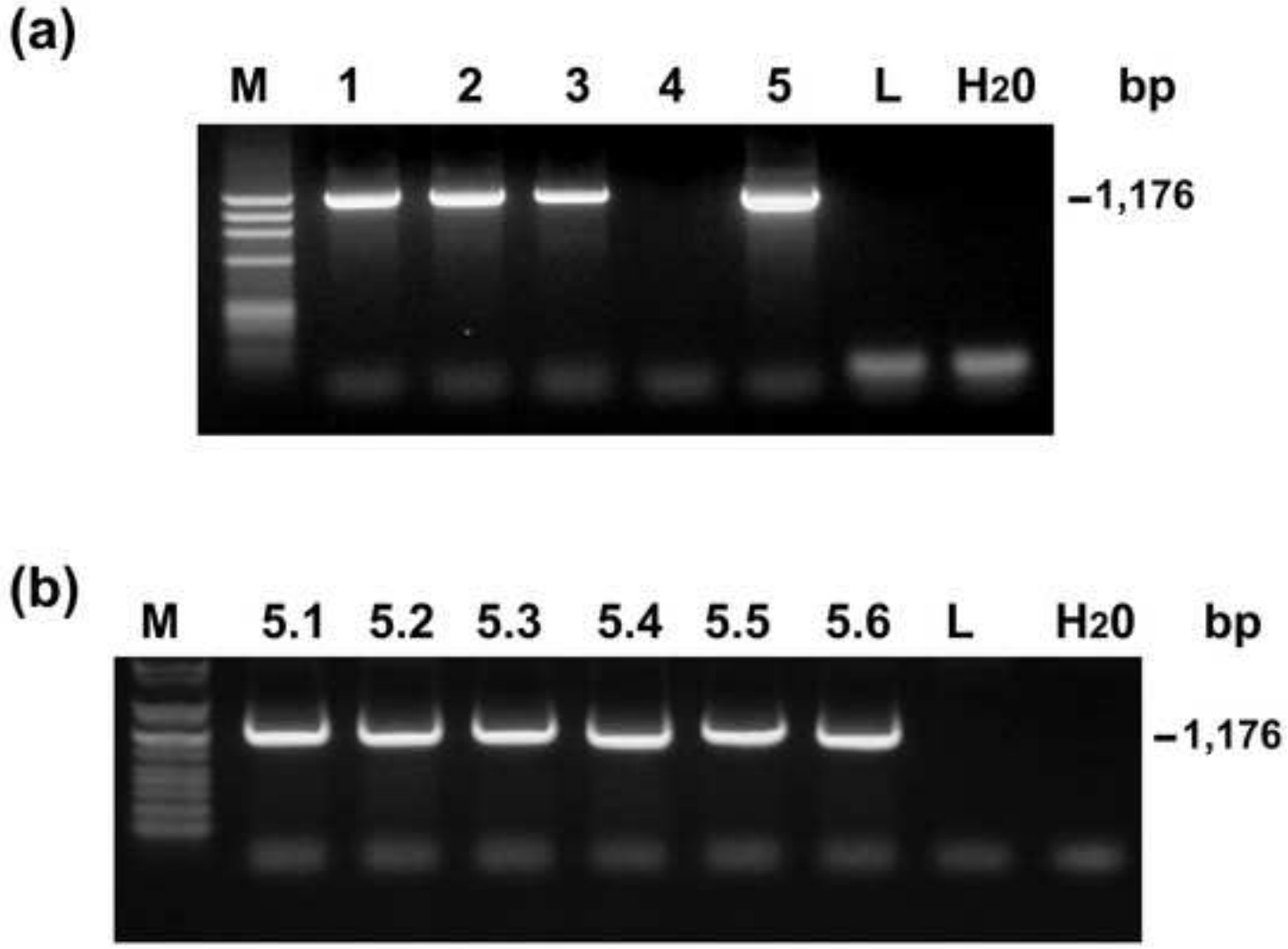

Fig. 4

Figure
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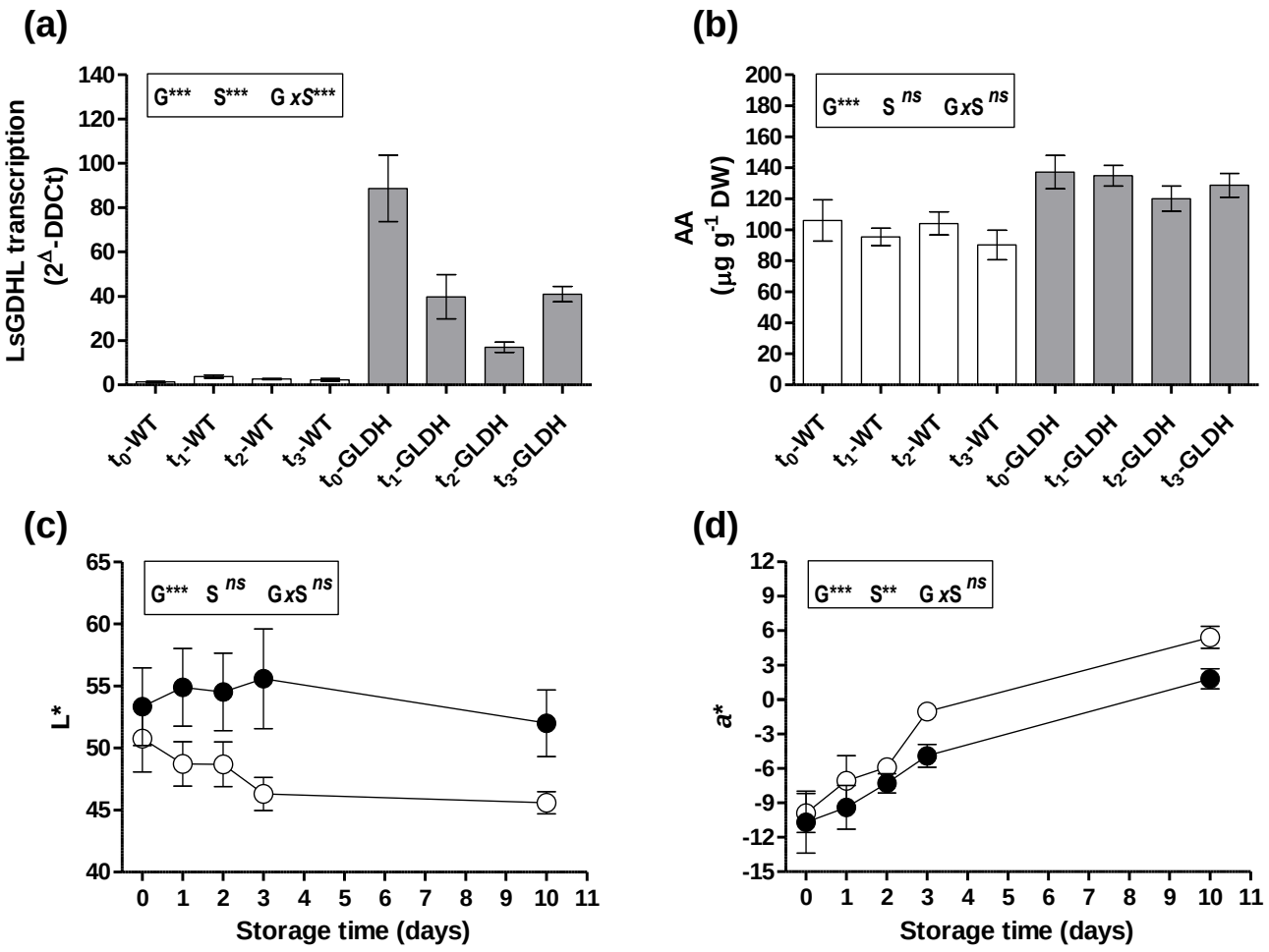

Online Resource 1

Plant Cell, Tissue and Organ Culture

Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browning in leaves of *Lactuca sativa* after cutting

Marco Landi, Marco Fambrini, Alice Basile, Mariangela Salvini, Lucia Guidi, Claudio Pugliesi*

*Corresponding author: Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy
Email: claudio.pugliesi@unipi.it

List of primers used for amplification of *GLDH* cDNA of *Lactuca sativa* (*LsGLDH*) and genetic transformation study

Use	Primer	Primer sequence 5'-3'
Amplification of cDNAs fragments	LAC1F	forward, 5'-GAGCCGATGTGATGAATCCCGGA-3'
	LAC4R	reverse, 5'-GCTTCATCGAGGCTATACTTGGGC-3'
3'-RACE	LAC5F	forward, 5'-TGGAGGTCAACAATGGGTCTCTG-3'
	UAP9	reverse, 5'-GACCACGCGTATCGATGTCGAC-3'

Full length isolation of *LsGLDH* cDNA	LAC1F	forward, 5’-GAGCCGATGTGATGAATCCCGGA-3’
	LAC6R	reverse, 5’-CCTTTTTAGTACTTGAATCCTCTTC-3’
Construction of *PetE::GLDH* cassette	LATF	forward, 5’-GGGTCGACATGTTGCGATCTCTCCGATTCC-3’
	LATR	reverse, 5’-GGGTCGACTTAAGTTGCATTATCTTCTATTGG-3’
Analysis of putative transgenic lettuce plants	PCPF	forward, 5’-GCCACGTCGGAGGATAACATCC-3’
	LAC4R	reverse, 5’-GCTTCATCGAGGCTATACTTGGGC-3’
Gene transcription analysis	Lsβtub3F	forward, 5’-CAGGATCAGGAATGGGAACTC-3’
	Lsβtub3R	reverse, 5’-CCTTGGGAGAAGGGAATACAG-3’
	LsGLDHF	forward, 5’-CCACTTACTACTCCTTCCCTTTC-3’
	LsGLDHR	reverse, 5’-TGAGTCCCGCTCCAATTAAC-3’

Online Resource 2

Plant Cell, Tissue and Organ Culture

Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browning in leaves of *Lactuca sativa* after cutting

Marco Landi, Marco Fambrini, Alice Basile, Mariangela Salvini, Lucia Guidi, Claudio Pugliesi*

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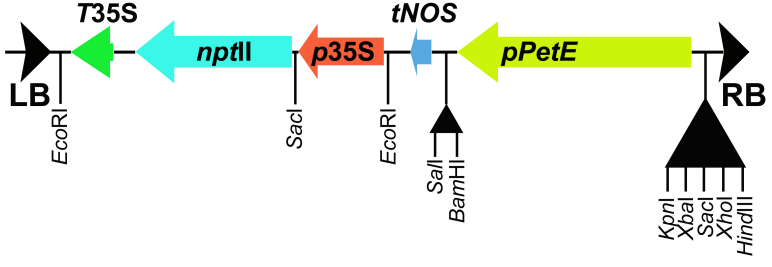
Pea plastocianin promoter *PetE-NOS* terminator expression cassette (Frugis et al. 2001)



Online Resource 3

Plant Cell, Tissue and Organ Culture

Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browning in leaves of *Lactuca sativa* after cutting

Marco Landi, Marco Fambrini, Alice Basile, Mariangela Salvini, Lucia Guidi, Claudio Pugliesi*

*Corresponding author: Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy
Email: claudio.pugliesi@unipi.it

Nucleotide sequences of *Lactuca sativa* L-galactono-1,4-lactone dehydrogenase (*LsGLDH*) (EMBL GenBank accession number HG810915.2). In bold characters and highlighted in green and magenta are the start and the stop codon, respectively. The region encoding the cleavage site Phenilalanine-Arginine/Tyrosine-Alanine (FR/YA) amino acids for mitochondrial targeting is in bold and red characters (see also Fig. 2A). Highlighted in light bleu is the region encoding the FAD-binding domain (Fraaije et al. 1998; Leferink et al. 2008). Highlighted in yellow is the codon for the Leucine (L) at position 161 of the LsGLDH amino acid sequence. Highlighted in gray are the codons for the Glutamic acid (E) and Arginine (R) at position 485 and 487 of the LsGLDH amino acid sequence, respectively. Double underlined is the region encoding the domain specific to D-arabinono-1,4-lactone oxidase

GAGCCGATGTGATGAATCCCGGAAAAATCAATACAA**ATG**TTGCGATCTCTCCGATTCCAGCGATCCCTCCAAT
CCTCCGTTATCCACCGCAAAAACCCACATTTCAACAACACCCTCGAAACCCTATCTTCTTCACCTA
CCACTAAAACCCCTCCAATAAACCTCATACGTCAATTTTCTTCATCATCTCCACCGCCGCCAACAC
CGCCGCCTCTATCCGCCACGCCATCTACTTCCTCTGAACTCCGGAAGTACCTCGGATACTCCGCAC
TCCTCCTCAGCTGCGCCGTCGCCACTTACTACTCCTTCCCTTTCCCTGAAAACGCAAAACACAAAA
AGGCCCAACTC**TTCCGGTACGCC**CCTATTCCCGATGACCTCCACACGGTCGTTAATTGGAGCGGGA
CTCACGAAGTACAGACCCGGGTCTTCCTCCAACCCGAAAGTTTAGAAGAATTGGAGAAGATTGTGA
AGGATGCCGACGAGAAGAAGCAGAAGATCCGTCCTGTTGGATCCGGGTTATCCCCGAATGGAATCG
GTTTGGCCCGTGGTGGGATGGTGAATCTGGCTTTGATGGATAAAGTTTTGGAGGTGGATAAAGAGA
AGAAAACGGTTCGGGTACAGGCAGGCATTCGAGTCCAGCAACTTGTCGATGTTGTCAAGGATCATG
GCATTACTTTGCAGAACTTTGCGTCTATCAGAGAACAACAAATTGGTGGCATTGTTCAGGTAGGTG

CTCATGGTACTGGTGCAAAGTTGCCTCCGATTGATGAGCAGGTTATCAGCATGAAATTGGTTACCC
CTGGAAAGGGAACAATCGAAATTTCAAAAGACAAAAACCCTGAACTGTTCTATTTAGCTCGATGCG
GGCTTGGTGCATTTGGGGTTGTTGCTGAAGTTACTCTCCAATGTGTTGAGAGACAGGAACTTGTAG
AACACACATTTGTCTCAAACTTGACTGAAATCAAGAAGAAACACAAAAAGCTTCTAAATGACAACA
AGCATGTAAAGTACCTTTACATACCATATACAGACACTGTTGTAGTGGTGACATGTAACCCTGTTT
CCAAATGGAAAGGCCCACCCAAATTTAAGCCCAAGTATAGCCTCGATGAAGCTTTACAACCTGTTC
GTGATCTATACAAAGAATCATTACAAAAGTACAAACGCCAACCAAATGAAAACGACTCAAAGGTTT
CAGACCTTACATTTACTGAACTAAGAGACAAGTTACTATCCATTGATCCTCTTAACAAAGACCATG
TTAAGAAAATCAATGAAGCAGAATCCGAATTTTGGAAGAGATCAGAGGGATTTAGGGTAGGGTGGA
GCGATGAAATTTTAGGGTTTGATTGTGGAGGTCAACAATGGGTCTCTGAGACATGTTTTCCAGCTG
GAACTTTATCAAAACCAAACATGAAAGATCTTAAATTTATAGAAGAAGTTATGGAATTAATAGAGA
AAGAAGAGATTCCTGCTCCTTCACCTATAGAACAAAGATGGTCTGCTTCTAGCAAAAGTTTGATGA
GTCCTGCTTCAAGTGAATCAAATGATGATATTTTCTCATGGGTGGGTATAATTATGTATCTTCCTA
CATCAGATGCTCGACAAAGGAAACAAATAACAGAAGAATTTTTTCATTATAGGCATCTCACTCAAA
CACGTTTTTGGAATCAATATTCAGCTTTTGAACATTGGGCCAAAATTGAGGTTCCAAAAGACAAAA
ACGAGCTTGCAGCCCTACAAGAAAGGCTAAGAGCACGATTCCCTGTTGATGCATTCAACAAAGCAC
GAAAAGAGTTGGATCCGAATCGTATTCTTTCCAATGCCATGGTGGAGAAGATGTTCCCAATAGAAG
ATAATGCAACT**TAA**TTACCAAAATTTATGTGTAAATTACATGAAGAGGATTCAAGTACTAAAAAGGATATCAAATTTT
AAAAATAATGTCTTCATATCCTACAAATAAAAAA

Online Resource 4

Plant Cell, Tissue and Organ Culture

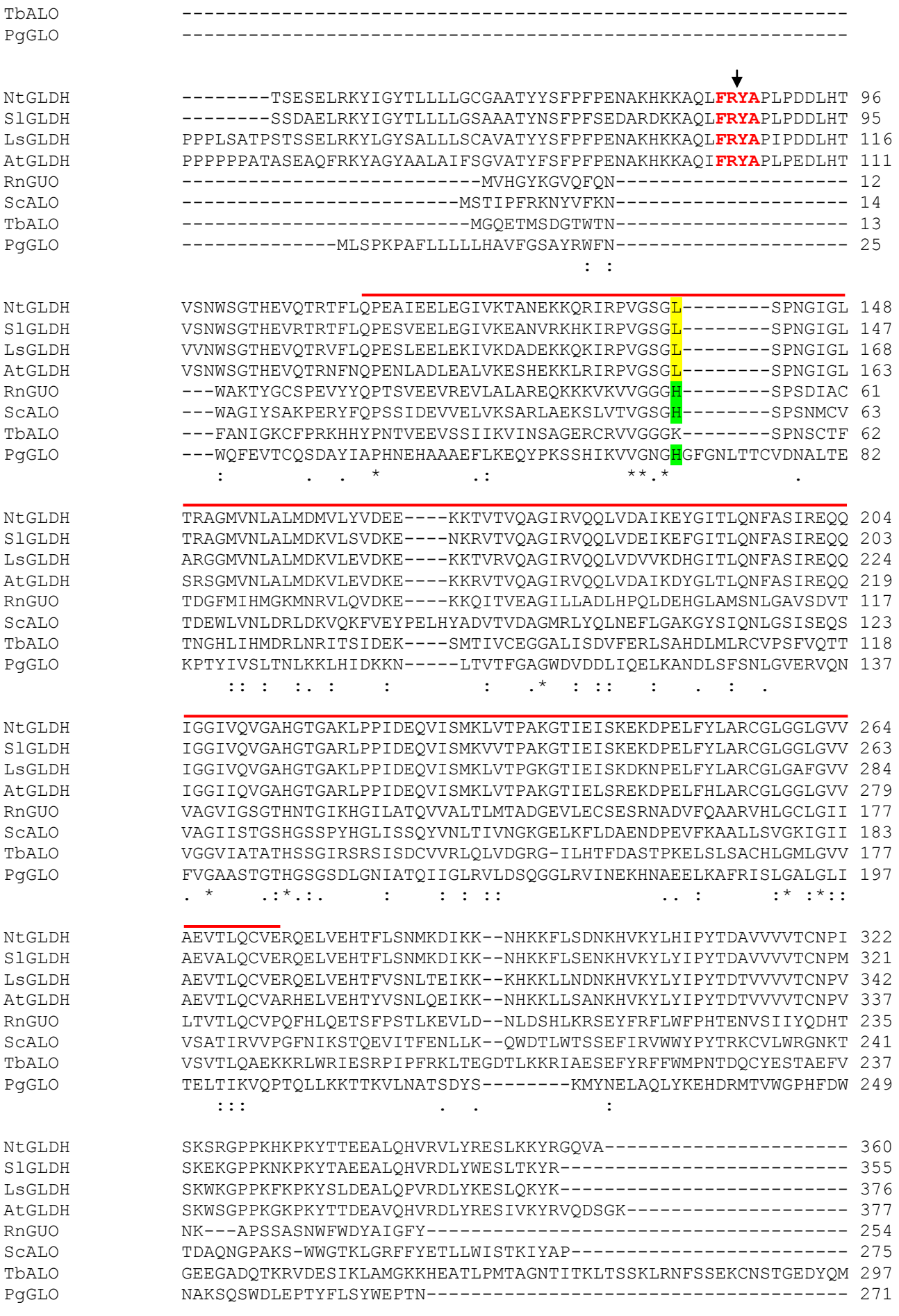
Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browning in leaves of *Lactuca sativa* after cutting

Marco Landi, Marco Fambrini, Alice Basile, Mariangela Salvini, Lucia Guidi, Claudio Pugliesi*

*Corresponding author: Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy
Email: claudio.pugliesi@unipi.it

Multiple sequence alignment of the full length amino acid sequence of LsGALDH with several aldonolactone oxidoreductases. The GenBank accession numbers (NCBI) used for the multiple sequence alignment are: NtGLDH, tobacco GLDH (AB024527); SlGLDH, tomato GLDH (HM587129); LsGLDH, lettuce GLDH (HG810915.2); AtGLDH, *Arabidopsis thaliana* GLDH (AB042279); RnGUO, rat GUO (P10867); ScALO, *Saccharomyces cerevisiae* ALO (P54783); PgGLO, *Penicillium griseoroseum* GLO (AAT80870); TbALO, *Trypanosoma brucei* ALO (AAX79383). Alignment was performed using CLUSTAL W. Amino acid residue numbers are shown on the right. The arrow indicates the putative cleavage site (red characters) of the mitochondrial targeting sequence in plant GLDH (FR↗YA). The histidine residue involved in covalent binding of the FAD cofactor in GUO, ALO and GLO are highlighted in green. In the same position, the leucine residues of plant GLDHs are highlighted in yellow. The arginine residues (R487 in LsGLDH), crucial for the stabilization of the anionic form of the reduced FAD cofactor, are highlighted in green (Leferink et al. 2009). The glutamic acid residues (E485 in LsGLDH), involved in productive substrate binding, are highlighted in gray (Leferink et al. 2009). The FAD-binding domain (Fraaije et al. 1998; Leferink et al. 2008) is red upperlined.

```
NtGLDH          MLRSLTSKR----SLQSLLHYHHHPLLRPNPHPTPFNP----------RPFSSTPGPT-- 44
SlGLDH          MLRSFASKR----SLQSLLHHHYR-RCRQNPQFPIFNP----------RPFSSSPGPP-- 43
LsGLDH          MLRSLRFQR----SLQSSVIHRKNPHFNNTLETLSSSPTTKTPPINLIRQFSSSSPPPPT 56
AtGLDH          MLRSLLLRRSVGHSLGTLSPSSSTIRSSFSPHRTLCTTG---------QTLTPPPPPPPR 51
RnGUO           ------------------------------------------------------------
ScALO           ------------------------------------------------------------
```


TbALO ----------
PgGLO ----------
NtGLDH --------TSESELRKYIGYTLLLLGCGAATYYSFPFPENAKHKKAQLFRYAPLPDDLHT 96
SlGLDH --------SSDAELRKYIGYTLLLLGSAAATYNSFPFSEDARDKKAQLFRYAPLPDDLHT 95
LsGLDH PPPLSATPSTSSELRKYLGYSALLLSCAVATYYSFPFPENAKHKKAQLFRYAPIPDDLHT 116
AtGLDH PPPPPPATASEAQFRKYAGYAALAIFSGVATYFSFPFPENAKHKKAQIFRYAPLPEDLHT 111
RnGUO ----------MVHGYKGVQFQN---------- 12
ScALO ----------MSTIPFRKNYVFKN---------- 14
TbALO ----------MGQETMSDGTWTN---------- 13
PgGLO ----------MLSPKPAFLLLLLHAVFGSAYRWFN---------- 25
NtGLDH VSNWSGTHEVQTRTFLQPEAIEELEGIVKTANEKKQRIRPVGSGL--------SPNGIGL 148
SlGLDH VSNWSGTHEVRTRTFLQPESVEELEGIVKEANVRKHKIRPVGSGL--------SPNGIGL 147
LsGLDH VVNWSGTHEVQTRVFLQPESLEELEKIVKDADEKKQKIRPVGSGL--------SPNGIGL 168
AtGLDH VSNWSGTHEVQTRNFNQPENLADLEALVKESHEKKLRIRPVGSGL--------SPNGIGL 163
RnGUO ---WAKTYGCSPEVYYQPTSVEEVREVLALAREQKKKVKVVGGGH--------SPSDIAC 61
ScALO ---WAGIYSAKPERYFQPSSIDEVVELVKSARLAEKSLVTVGSGH--------SPSNMCV 63
TbALO ---FANIGKCFPRKHHYPNTVEEVSSIIKVINSAGERCRVVGGGK--------SPNSCTF 62
PgGLO ---WQFEVTCQSDAYIAPHNEHAAAEFLKEQYPKSSHIKVVGNGHGFGNLTTCVDNALTE 82
NtGLDH TRAGMVNLALMDMVLYVDEE----KKTVTVQAGIRVQQLVDAIKEYGITLQNFASIREQQ 204
SlGLDH TRAGMVNLALMDKVLSVDKE----NKRVTVQAGIRVQQLVDEIKEFGITLQNFASIREQQ 203
LsGLDH ARGGMVNLALMDKVLEVDKE----KKTVRVQAGIRVQQLVDVVKDHGITLQNFASIREQQ 224
AtGLDH SRSGMVNLALMDKVLEVDKE----KKRVTVQAGIRVQQLVDAIKDYGLTLQNFASIREQQ 219
RnGUO TDGFMIHMGKMNRVLQVDKE----KKQITVEAGILLADLHPQLDEHGLAMSNLGAVSDVT 117
ScALO TDEWLVNLDRLDKVQKFVEYPELHYADVTVDAGMRLYQLNEFLGAKGYSIQNLGSISEQS 123
TbALO TNGHLIHMDRLNRITSIDEK----SMTIVCEGGALISDVFERLSAHDLMLRCVPSFVQTT 118
PgGLO KPTYIVSLTNLKKLHIDKKN-----LTVTFGAGWDVDDLIQELKANDLSFSNLGVERVQN 137
NtGLDH IGGIVQVGAHGTGAKLPPIDEQVISMKLVTPAKGTIEISKEKDPELFYLARCGLGGLGVV 264
SlGLDH IGGIVQVGAHGTGARLPPIDEQVISMKVVTPAKGTIEISKEKDPELFYLARCGLGGLGVV 263
LsGLDH IGGIVQVGAHGTGAKLPPIDEQVISMKLVTPGKGTIEISKDKNPELFYLARCGLGAFGVV 284
AtGLDH IGGIIQVGAHGTGARLPPIDEQVISMKLVTPAKGTIELSREKDPELFHLARCGLGGLGVV 279
RnGUO VAGVIGSGTHNTGIKHGILATQVVALTLMTADGEVLECSESRNADVFQAARVHLGCLGII 177
ScALO VAGIISTGSHGSSPYHGLISSQYVNLTIVNGKGELKFLDAENDPEVFKAALLSVGKIGII 183
TbALO VGGVIATATHSSGIRSRSISDCVVRLQLVDGRG-ILHTFDASTPKELSLSACHLGMLGVV 177
PgGLO FVGAASTGTHGSGSDLGNIATQIIGLRVLDSQGGLRVINEKHNAEELKAFRISLGALGLI 197
NtGLDH AEVTLQCVERQELVEHTFLSNMKDIKK--NHKKFLSDNKHVKYLHIPYTDAVVVVTCNPI 322
SlGLDH AEVALQCVERQELVEHTFLSNMKDIKK--NHKKFLSENKHVKYLYIPYTDAVVVVTCNPM 321
LsGLDH AEVTLQCVERQELVEHTFVSNLTEIKK--KHKKLLNDNKHVKYLYIPYTDTVVVVTCNPV 342
AtGLDH AEVTLQCVARHELVEHTYVSNLQEIKK--NHKKLLSANKHVKYLYIPYTDTVVVVTCNPV 337
RnGUO LTVTLQCVPQFHLQETSFPSTLKEVLD--NLDSHLKRSEYFRFLWFPHTENVSIIYQDHT 235
ScALO VSATIRVVPGFNIKSTQEVITFENLLK--QWDTLWTSSEFIRVWWYPYTRKCVLWRGNKT 241
TbALO VSVTLQAEKKRLWRIESRPIPFRKLTEGDTLKKRIAESEFYRFFWMPNTDQCYESTAEFV 237
PgGLO TELTIKVQPTQLLKKTTKVLNATSDYS--------KMYNELAQLYKEHDRMTVWGPHFDW 249
NtGLDH SKSRGPPKHKPKYTTEEALQHVRVLYRESLKKYRGQVA---------- 360
SlGLDH SKEKGPPKNKPKYTAEEALQHVRDLYWESLTKYR---------- 355
LsGLDH SKWKGPPKFKPKYSLDEALQPVRDLYKESLQKYK---------- 376
AtGLDH SKWSGPPKGKPKYTTDEAVQHVRDLYRESIVKYRVQDSGK---------- 377
RnGUO NK---APSSASNWFWDYAIGFY---------- 254
ScALO TDAQNGPAKS-WWGTKLGRFFYETLLWISTKIYAP---------- 275
TbALO GEEGADQTKRVDESIKLAMGKKHEATLPMTAGNTITKLTSSKLRNFSSEKCNSTGEDYQM 297
PgGLO NAKSQSWDLEPTYFLSYWEPTN---------- 271

```
                          .

NtGLDH          -------DSGSP-EPEIDELSFTELRDKLLALDPLNKVHVIEINKAEVEFWRKSEGYRVG 412
SlGLDH          -------DSGSPSEPEIVELSFTELRDKLLAMDPLNKEHVIKVNKAEAVYWRKSEGYRVG 408
LsGLDH          -------RQPNENDSKVSDLTFTELRDKLLSIDPLNKDHVKKINEAESEFWKRSEGFRVG 429
AtGLDH          -------KSPDSSEPDIQELSFTELRDKLLALDPLNDVHVAKVNQAEAEFWKKSEGYRVG 430
RnGUO           ------------------------LLEFLLWTSTYLPCLVGWINRFFFWMLFNCKKESSN 290
ScALO           ----------------------LTPFVEKFVFNRQYGKLEKSSTGDVNVTDSISGFNMDCL 314
TbALO           WLRNQRTLRTRICKILKGSWLRHGVVEAALAAAVIQPGIQPYINRTYRRLFYNAPEVQYG 357
PgGLO           ---------------------YTGVRNCTLNYCANGCGDCKKEYICYDEVTDAASCSPQG 310
                                     . :  :

NtGLDH          WSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEYIEELMQLIEKE-SVPAPAPIEQRW 471
SlGLDH          WSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEYIEELMQLIEKE-SVPAPAPIEQRW 467
LsGLDH          WSDEILGFDCGGQQWVSETCFPAGTLSKPNMKDLKFIEEVMELIEKE-EIPAPSPIEQRW 488
AtGLDH          WSDEILGFDCGGQQWVSESCFPAGTLANPSMKDLEYIEELKKLIEKE-AIPAPAPIEQRW 489
RnGUO           LSHKIFTYECRFKQHVQDWAIPR-------EKTKEALLELKAMLEAHPKVVAHYPVEVRF 343
ScALO           FSQFVDEWGCPMDNGLEVLRSLDHSIAQAAINKEFYVHVPMEVRCSNTTLPSEPLDTSKR 374
TbALO           TSLECFTFDCLFKQWACEWAIDIS-------NVMPAFHYLRGLISSE-NLSVHFPVEFRF 409
PgGLO           VCSRGFYAEIEHFLPIEYFAEAATN---------YTIFQQGQTSRMKAPYNKQMVMQHRS 361
                 .                                  .        .          :

NtGLDH          TACS------------------KSRMSPAYSSADDDIFSWVGIIMYLPTMDARQRRQITE 513
SlGLDH          TACS------------------KSRMSPAYSSADDDIFSWVGIIMYLPTMDARQRRQITE 509
LsGLDH          SASS------------------KSLMSPASSESNDDIFSWVGIIMYLPTSDARQRKQITE 530
AtGLDH          TARS------------------KSPISPAFSTSEDDIFSWVGIIMYLPTADPRQRKDITD 531
RnGUO           TRGD------------------DILLSPCFQRDS----CYMNIIMYRPYGKDVPR----- 376
ScALO           TNTSPGPVYGNVCRPFLDNTPSHCRFAPLENVTNSQLTLYINATIYRPFGCNTPIHKWFT 434
TbALO           TGAD------------------TAALSPAHGRQT----CWIGIVMYRPYLRHARDT---- 443
PgGLO           LKGD------------------DTYLSPVNTYNLGPDLSGVFGVIEIDWIQEYNNFTTLW 403
                   .                      ::*           :   :

NtGLDH          EFFHYRHMT------QAQLWDHYSAFEHWAKIEVPKDKEELAALQERLKKKFP--VDAYN 565
SlGLDH          EFFHYRHMT------QSQLWDQYSAFEHWAKIEVPKDKEELAALQARLKKKFP--VDAYN 561
LsGLDH          EFFHYRHLT------QTRFWNQYSAFEHWAKIEVPKDKNELAALQERLRARFP--VDAFN 582
AtGLDH          EFFHYRHLT------QKQLWDQFSAYEHWAKIEIPKDKEELEALQARIRKRFP--VDAYN 583
RnGUO           ---LDYWLA------YETIMKKFGGRPHWAKAHN--------CTQKDFEEMYPT-FHKFC 418
ScALO           LFENTMMVAGGKPHWAKNFLGSTTLAAGPVKKDTDYDDFEMRGMALKVEEWYGEDLKKFR 494
TbALO           ---LRYYDA------FSKAMTVMGGRPHWAKYYT--------WGPEQMLKAYGKNWEDFL 486
PgGLO           QNQELAHEF------LPQFGETYNARSHWNKMSAP--------NATYTLEKFPK-LPEFL 448
                                          *                    :      :

                QARKELDPNRILSN----NMLEKLFI---------- 587
NtGLDH          QARKELDPNRILSN----NMLEKLFPSSEAV----- 588
SlGLDH          KARKELDPNRILSN----AMVEKMFPIEDNAT---- 610
LsGLDH          KARRELDPNRILSN----NMVEKLFPVSTTA----- 610
AtGLDH          DIREKLDPTGMFLN----SYLEKVFY---------- 440
RnGUO           KIRKEQDPDNVFLANKQWAIINGIIDPSELSD---- 526
ScALO           LFRKKMDPEGVFLNGWFNSLSGNSPVLNSTISHL-- 520
TbALO           AIQKRQDPKCQFVN----EFLVEQLGITRCANYISV 480
PgGLO             :.. **   :
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Online Resource 5

Plant Cell, Tissue and Organ Culture

Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browning in leaves of *Lactuca sativa* after cutting

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List of 14 amino acid sequences of L-galactono-1,4-lactone dehydrogenase (GLDH) used for the phylogenetic analysis (Fig. 2b). The GenBank accession numbers are in brackets

Lactuca sativa (HG810915.2)

MLRSLRFQRSLQSSVIHRKNPHFNNTLETLSSSPTTKTPPINLIRQFSSSSPPPPTPPPLSATPSTSSELRKYLGYSALL
LSCAVATYYSFPFPENAKHKKAQLFRYAPIPDDLHTVVNWSGTHEVQTRVFLQPESLEELEKIVKDADEKKQKIRPVGSG
LSPNGIGLARGGMVNLALMDKVLEVDKEKKTVRVQAGIRVQQLVDVVKDHGITLQNFASIREQQIGGIVQVGAHGTGAKL
PPIDEQVISMKLVTPGKGTIEISKDKNPELFYLARCGLGAFGVVAEVTLQCVERQELVEHTFVSNLTEIKKKHKKLLNDN
KHVKYLYIPYTDTVVVVTCNPVSKWKGPPKFKPKYSLDEALQPVRDLYKESLQKYKRQPNENDSKVSDLTFTELRDKLLS
IDPLNKDHVKKINEAESEFWKRSEGFRVGWSDEILGFDCGGQQWVSETCFPAGTLSKPNMKDLKFIEEVMELIEKEEIPA
PSPIEQRWSASSKSLMSPASSESNDDIFSWVGIIMYLPTSDARQRKQITEEFFHYRHLTQTRFWNQYSAFEHWAKIEVPK
DKNELAALQERLRARFPVDAFNKARKELDPNRILSNAMVEKMFPIEDNAT

Camellia sinensis (KF619448)

MFRALNIRRSLYQALHHHHHHHHHHHHHHHHHPLSQPLKTLSSTQNPNLTRPFCSSTSSSSSEAEFRKYVGYFALLVGCGI
ATYYSFPFPENAKHKKAQLFRYAPLPDDLHTVSNWSGTHEVQTRVFLQPESIQELETIVRDANVQKQKIRPVGSGLSPNG
IGLTRLGMVNLALLDRVLEVDKEKKTVRVEAGIRVQQLVDGIKDYGLTLQNFASIREQQIGGIVQVGAHGTGARLPPIDE
QVVSMKLVTPAKGTIEVSKEKDPELFYLARCGLGALGVVAEVTLQCVERQELVEHTFVSNTEEIKKNHKKFLSENKHVKY
LYIPYTDTVVVVRCNPVSKWKGPPKFKPKYSHDEAMQNVRDLYQESLKKYRRAVTTTESVDNNEQDINDLSFTELRDKLL
ALDPLNKNHIIKVNQAEAEFWRKSEGYRVGWSDEILGFDCGGQQWVSETCFPAGTLSKPSMKDLEYIEELMQLIDKEAIP
APAPIEQRWTARSKSLMSPASSTADDDIFSWVGIIMYLPTMDARQRKEITDEFFHYRHLSQSQLWDRYSAYEHWAKIEVP
KDKDELATLQARLRKRFPVDAYNKARRELDPNHILSNNMLEKLFPQSDII

Ipomoea batatas (AB017357)

MFRAHHFRRSLRSLLAHSHSHPHSNPHINPRLLCSLSSQPPSSDAEVRKYIGYTVLVLGCAAATYYSFPFPADAKHKKAQ
LFRYAPLPDDLHTVTNWSGTHEVQTRTFLQPESLQELEAAVKDSNEKKQKIRPVGSGLSPNGIGLTRAGMVNLGLMDKVL
EVDKEKKRVTVQAGIRVQQLVDSIKEYGLTLQNFASIREQQVGGIVQVGAHGTGARLPPIDEQVISMKLVTPAKGTIEIS
KEKDPDLFYLARCGLGGLGVVAEVTLQCVERQELVEHTYISNMKDIKKNHKKLLSENKHVKYLHIPYTDAVVVVTCNPIS

KWKGPPKYKPKYSPEEAVGHVQDLYRESLKKYRSTENESEINELSFTELRDKLLALDPLNTDHVKKTNQAEAEFWRKSEG
YRVGWSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEFIEQLMQLIEKESIPAPAPIEQRWTACSKSLMSPAYSSVDD
DIFSWVGIIMYLPTMDARERKHITEEFFHYRHLTQAHLWDHYSAYEHWAKIEVPKDKEELQALQARLRKKFPVDAYNRAR
QELDPNRILSNNMLEKLFPSS

Nicotiana tabacum (AB024527)

MLRSLTSKRSLQSLLHYHHHPLLRPNPHPTPFNPRPFSSTPGPTTSESELRKYIGYTLLLLGCGAATYYSFPFPENAKHK
KAQLFRYAPLPDDLHTVSNWSGTHEVQTRTFLQPEAIEELEGIVKTANEKKQRIRPVGSGLSPNGIGLTRAGMVNLALMD
MVLYVDEEKKTVTVQAGIRVQQLVDAIKEYGITLQNFASIREQQIGGIVQVGAHGTGAKLPPIDEQVISMKLVTPAKGTI
EISKEKDPELFYLARCGLGGLGVVAEVTLQCVERQELVEHTFLSNMKDIKKNHKKFLSDNKHVKYLHIPYTDAVVVVTCN
PISKSRGPPKHKPKYTTEEALQHVRVLYRESLKKYRGQVADSGSPEPEIDELSFTELRDKLLALDPLNKVHVIEINKAEV
EFWRKSEGYRVGWSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEYIEELMQLIEKESVPAPAPIEQRWTACSKSRMS
PAYSSADDDIFSWVGIIMYLPTMDARQRRQITEEFFHYRHMTQAQLWDHYSAFEHWAKIEVPKDKEELAALQERLKKKFP
VDAYNQARKELDPNRILSNNMLEKLFI

Malus domestica (FJ752244)

MQRALTLRRSVESLHHHNHLRAITPTTVPQNPLTQVSSTRALSTLSSPSSPAPSSSSSELRKYLGYTALVLFSAAATYYS
FPFPENAKHKKAQIFRYAPLPEDLHTVSNWSGTHEVQTRVFHQPETLEELEKVVKDAHEKKSRIRPVGSGLSPNGIGLSR
AGMVNLALMDKVLEVDKEKKRVRVQAGIRVQQLVDGIKEHGITLQNFASIREQQIGGILQVGAHGTGARLPPMDEQVISM
KLVTPAKGTIEVSKEKDPELFYLARCGLGGLGVVAEVTIQCVDRQELVEHTTVSTMAEIKKNHKKLLSENRHVKYLYIPY
TDTVVVVKCNPVSKWKGPPKFTPKYSSDEAIQHVRDLYRECLQKYRVVPDKSEVDMNELSFTELRDKLLALDPLDKDHVA
KVNQAEAEFWKKSEGYRVGWSDEILGFDCGGQQWVSETCFPAGTLAKPSMKDLEYIEGVKQLIEKNEIPAPAPIEQRWAA
CTRRPMSPASSTREDDILSWVGIIMYLPTTDPRQRKEITEEFFHYRRLTQTQLWDKYSAYEHWAKIEVPKDKDELAALQD
RLRKRFPVDAYNKARRELDPNRVLSNSKLEKLFPLSDTI

Solanum lycopersicum (HM587129)

MLRSFASKRSLQSLLHHHYRRCRQNPQFPIFNPRPFSSSPGPPSSDAELRKYIGYTLLLLGSAAATYNSFPFSEDARDKK
AQLFRYAPLPDDLHTVSNWSGTHEVRTRTFLQPESVEELEGIVKEANVRKHKIRPVGSGLSPNGIGLTRAGMVNLALMDK
VLSVDKENKRVTVQAGIRVQQLVDEIKEFGITLQNFASIREQQIGGIVQVGAHGTGARLPPIDEQVISMKVVTPAKGTIE
ISKEKDPELFYLARCGLGGLGVVAEVALQCVERQELVEHTFLSNMKDIKKNHKKFLSENKHVKYLYIPYTDAVVVVTCNP
MSKEKGPPKNKPKYTAEEALQHVRDLYWESLTKYRDSGSPSEPEIVELSFTELRDKLLAMDPLNKEHVIKVNKAEAVYWR
KSEGYRVGWSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEYIEELMQLIEKESVPAPAPIEQRWTACSKSRMSPAYS
SADDDIFSWVGIIMYLPTMDARQRRQITEEFFHYRHMTQSQLWDQYSAFEHWAKIEVPKDKEELAALQARLKKKFPVDAY
NQARKELDPNRILSNNMLEKLFPSSEAV

Arabidopsis thaliana (AB042279)

MLRSLLLRRSVGHSLGTLSPSSSTIRSSFSPHRTLCTTGQTLTPPPPPPPRPPPPPPATASEAQFRKYAGYAALAIFSGV
ATYFSFPFPENAKHKKAQIFRYAPLPEDLHTVSNWSGTHEVQTRNFNQPENLADLEALVKESHEKKLRIRPVGSGLSPNG
IGLSRSGMVNLALMDKVLEVDKEKKRVTVQAGIRVQQLVDAIKDYGLTLQNFASIREQQIGGIIQVGAHGTGARLPPIDE
QVISMKLVTPAKGTIELSREKDPELFHLARCGLGGLGVVAEVTLQCVARHELVEHTYVSNLQEIKKNHKKLLSANKHVKY
LYIPYTDTVVVVTCNPVSKWSGPPKGKPKYTTDEAVQHVRDLYRESIVKYRVQDSGKKSPDSSEPDIQELSFTELRDKLL
ALDPLNDVHVAKVNQAEAEFWKKSEGYRVGWSDEILGFDCGGQQWVSESCFPAGTLANPSMKDLEYIEELKKLIEKEAIP
APAPIEQRWTARSKSPISPAFSTSEDDIFSWVGIIMYLPTADPRQRKDITDEFFHYRHLTQKQLWDQFSAYEHWAKIEIP
KDKEELEALQARIRKRFPVDAYNKARRELDPNRILSNNMVEKLFPVSTTA

Fragaria x ananassa (AY102631)

MQRALTLKRTLQSLPRITKNPLISGRAFCNASTPSPSPASASELRKYMGYTALVLFCGAATYYSFPFPEDAKHKKAQIFR
YAPLPEELHTVSNWSGTHEVQTRVFHQPETLEELEKVVKEANARKYRIRPVGSGLSPNGIGLSRAGMVNLALMDEVLEVD
REKKRVRVQAGIRVQQLVDGIKDQGLTLQNFASIREQQIGGILQVGAHGTGARLPPIDEQVISMKLVTPAKGTIEVSKEK
DPELFYLARCGLGGLGVVAEVTLQCVERQELVEHTTVSNMEENKKNHKKLFSENKHVKYLYIPYTDTVVIVTCNPVSKWK
GPPKFKPKFTTDEAIQHVRDLYRDCLRKYRVVPDNSVDVDEPSFTELRGKLIALNPLNKDHIVKMNQAEAEFWRKSEGYR
VGWSDEILGFDCGGQQWVSETCFPAGTIAKPSMKDLEYIEDLKQLIEKEEIPAPAPIEQRWTASSKSPMSPASSLKGDNI
FSWVGIIMYLPTTDARQRKDITEEFFHYRHLTQTRLWDTYSSYEHWAKIEVPKDKEQLTALRARLRKRYPVDAYNKARSE
LDPSRILSNVKLGKLFPSSDTI

Cucumis sativus (HQ446099)

MLNFLSLRRSIHYFRHRRLQISPTAAISKPPFNPPRPFSTVSPSPSSSSFDSELRKYLGYGALVIFCGAATYYSFPFPEN
AKHKKAQIFRYAPLPEDLHTVSNWSGTHEVWTRVFHQPENLEQLEQVVKQANEKKARIRPVGSGLSPNGIGLSRMGMVNL
ALMDKVLEVDKEKKRVRVQAGIRVQQLVDGIKEYGLTLQNFASIREQQIGGIIQVGAHGTGAKLPPIDEQVIAMKLVTPA
KGTIEISKDKDPELFLLARCGLGGLGVVAEVTLQCVERQELVEHTYISNMKDIKKNHKKLLADNKHVKYLYIPYTDAIVV
VTCNPISKWRGPPKFKPKYTSEEAIQHVRDLYVESLKKYSASEERDMNEISFTELRDKLLALDPLNKEHVIKVNQAEAEF
WRKSEGYRVGWSDEILGFDCGGQQWVSETCFPAGTLAKPNMKDIEYIEELKQLIEKKNIPAPAPLEQRWTARSKSPMSPA
SSTAEDDIFSWVGIIMYLPTSDARQRKEITEEFFHYRHLTQTLLWDQYSAFEHWAKIEVPKDKDELAALQARLRKRFPVD
EYNKARRALDPNKILSNNKLEKLFSSTDTV

Capsicum annuum (AY547352)

MLRSFISKRSLQSFLRYHHHHHHRAHRRPFSTTPGPPTADAELRKYIGYTLLLVASGAATYYSFPFPENARDKKAQLFRY
APLPDDLHTVTNWSGTHEVRTRTFLQPESIEQLEGIVKEGHERKHKIRPVGSGLSPNGIGLTRAGMVNLALMDKVLSVDK
EKKRVTVQAGIRVQQLVDEIKEYGITLQNFASIREQQIGGIVQVGAHGTGARLPPIDEQVISMKLVTPAKGTIEISKEKD
PELFYLARCGLGGLGVVAEVTLQCVERQELVEHTFLSNMKDIKKNHKKFLSENKHVKYLHIPYTDAVVVVTCNPVSKLRG
PPKHKPIYTTEEALQHVRDLYQESLKKYRSQVAASGSPDEPEVDELSFTELRDKLLVMDPLNKEHVIKVNKAEAEYWRKS
EGYRVGWSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEYIEELMQLIEKESVPAPAPIEQRWTACSKSQMSPAYSSA
DDDIFSWVGIIMYLPTMDARQRKQITEEFFHYRHMTQAQLWDRYSAFEHWAKIEVPKDKEELAALQARLKKKFPVDAYNQ
ARNELDPNHILSNNMLEKLFPSSEAQ

Solanum tuberosum (FJ755844)

MLRSFTSKRSLQSLLHHRRCRQNPQFPIFNPRLFSSSPGPPSSDAELRKYIGYTLLVLGSAAATYYSFPFSENARDKKAQ
LFRYAPLPDDLHTVSNWSGTHEVRTRTFLQPESIEDLEGIVKEANVRKHKIRPVGSGLSPNGIGLTRAGMVNLALMDKVL
SVDKEKKRVTVQAGIRVQQLVDEIKEFGITLQNFASIREQQIGGIVQVGAHGTGARLPPIDEQVISMKLVTPAKGTIEIS
KEKDPELFYLARCGLGGLGVVAEVTLQCVERQELVEHTFLSNMKDIKKNHKKFLSENKHVKYLYIPYTDAVVVVTCNPMS
KRKGPPKNKPKYTTEEALQHVRDLYLESLTKYRGQVTDSGSPDEPEIVELSCTELRDKLLAMDPLNKEHVIKVNKAEAEY
WRKSEGYRVGWSDEILGFDCGGHQWVSETCFPAGTLSKPSMKDLEYIEELMQLIEKESVPAPAPIEQRWTACSKSRMSPA
YSSVDDDIFSWVGIIMYLPTMDARQRRQITEEFFHYRHMTQAQLWDHYSAFEHWAKIEVPKDKEELTALQARLKKKFPVD
AYNQARKELDPNRILSNNMLEKLFPSSEAV

Ricinus communis (XM_002531455)

MLRFLSLRRSLHHHHASKPLNSSSTLKHPFNPARTLSTSSTSSTSSTSSSSSLSDAELRKYLGYTALLLFSGAATYYSFP
FSDTAKHKKAQIFRYAPLPEDLHTVSNWSGTHEVQTRDFHQPEDLHQLEELVKDSNEKRAKIRPVGSGLSPNGIGLARGG
MVNLGLMDKVLEVDQEKKRVRVEAGIRVQELVDGIKDFGITLQNFASIREQQIGGIIQVGAHGTGARLPPIDEQVISMKL
VTPAKGTIEISKEKDPELFYLARCGLGGLGVVAEVTLQCVERQELVEHTYISNMKDIKKNHKKLLSENKHVKYLYIPYTD
SVVVVTCNPVSKWKGPPKFKPKYSQDEAIQHVRDLYKESLEKYRTGVVAGKSVDNDEMDINELSFTELRDKLLALAPLNK
DHVIKVNLAEAEFWRKSEGYRVGWSDEILGFDCGGQQWVSETCFPAGTLSKPSMKDLEYIEELKQLIEKEEIPAPAPIEQ
RWTARSQSSMSPASSSAEDDIFSWVGIIMYLPTMDARQRKDITEEFFHYRHLTQAQLWDKYSCFEHWAKIEVPKDKEEIA
ALQARLRKRFPVDAYNKARKELDPNRILSNNILEKLFPLSDTI

Malpighia glabra (EU683445)

MFRFITLNRTLRHQYNHRKTLIPAVQLKPTPTRTFCTSPPTATDSEVRKYLGYTALFIFCGAATYYSFPFSENAKHKKAQ
IFRYAPLPEDLHTVSNWSGTHEVQTRNFHQPETINELEELVKVSNEKKERIRPVGSGLSPNGIGLSRLGMVNLALVDKVL
EVDKEKKRVRVQAGIRVQELVDGIKEHGLTLQNFASIREQQIGGIVQVGAHGTGARLPPIDEQVISMKLVTPAKGTIEIS
KDKDPELFYLARCGLGGLGVVAEVTLQCVERQQLVEHTYISNMKDIRKNHKKLLSDNKHVKYLYIPYTDAVVVVTCNPVS
KWRGVPKFTPKYTEDEALQHVRDLYQEPLNKYRGGEITSKSSEDDSPDINELSFTELRDKLLALDPLNKDHVIKVNQAEA
EFWRKSEGYRVGWSDEILGFDCGGQQWVSETCFPAGTLANPSMQDLDYIEDLKQLIEKEDIPAPAPIEQRWTARSQSSMS
VASSSKEDDIFSWVGIIMYLPTMDARQRKEITEEFFHYRHLTQAELWDKYSAYEHWAKIEVPKDKEELEALLERLKKRFP
VDAYNKARKELDPNKILSNNKLEKLFPSLDAI

Volvox carteri (XM_002947966)

MTPGASGIFFAPRLQPHSRRGYGGVAGIPRGVLGGGGGEVAGAYVPPAAGGVGGAQHRDSPTRRAVGNFLRVLLPVSGIA
VWTRYFQPVSEEEVEAFLHIASVRGETLRPAGSGLSPNGLALSGEGVLALGAMDRVLRVDKNKMQVTVQAGARVQQVVEA
LAPQGLALQNYASIREQQIGGITQVGAHGTGPRIPPVDEQVVDMRLSTPGLGTLQLSDEEEPELFRLARVGLGSLGVMTE
ATLRVVPREPLIERTFTASRAEVHRNHVKWLQQNKHIKYLYIPYTDTVVVVQVNPPRTPEELQAAREEAAKPAHPEAERT
HALRRLYATVAAPESAPTASTTISATAPAPDTAAPTDPWWVAAVNAAEAEYWRRSAGVRVGFSDDLLAFDCGGQQWVLEV
AFPVAASLDGLKPGARTRDLEFLEALMAEIKKARLPAPSPIEVRWTSGSSSPLSPAAGPPESVHCWVGIIMYLPEEPEAR

EKVTQAFRGYTRLVESKLMPRFDATWHWAKLETSSRPEGELEGLVRPRLASRFGSALGALSRYRAVLDPQGTLANKWLDA
VLGPVPKQQQQAQERQAQE

Online Resource 6

Plant Cell, Tissue and Organ Culture

Overexpression of *L-galactono-1,4-lactone dehydrogenase* (*GLDH*) gene correlates with increased ascorbate concentration and reduced browing in leaves of *Lactuca sativa* after cutting

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Regeneration of *GLDH*-transformed plants of lettuce (*Lactuca sativa* L. cv' Iceberg'). **a** Petri dish with regenerated lettuce shoots. **b** A rooted lettuce shoot on medium supplemented with kanamycin. **c** Lettuce shoots on rooted medium. **d** Plants transplanted on soil in acclimation growth chamber. **e** A *PetE-GLDH* heterozygous lettuce plant at the anthesis stage. **f** Wild type (WT) and *PetE-GLDH* homozygous T2 plants