Plant Science 205–206 (2013) 63–75

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Sorbitol dehydrogenase is a cytosolic protein required for sorbitol metabolism in *Arabidopsis thaliana*

María Francisca Aguayo[a], Diego Ampuero[a], Patricio Mandujano[a,1], Roberto Parada[a], Rodrigo Muñoz[a], Marta Gallart[b,c], Teresa Altabella[b,c], Ricardo Cabrera[a], Claudia Stange[a], Michael Handford[a,*]

[a] *Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile*
[b] *Unitat de Fisiologia Vegetal, Facultat de Farmàcia, Universitat de Barcelona, Spain*
[c] *Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Campus UAB Bellaterra, Barcelona, Spain*

ARTICLE INFO

Article history:
Received 26 October 2012
Received in revised form 11 January 2013
Accepted 15 January 2013
Available online 7 February 2013

Keywords:
Alcohol dehydrogenase
Compatible solute
Dehydration
Sorbitol
Xylitol

ABSTRACT

Sorbitol is converted to fructose in Rosaceae species by SORBITOL DEHYDROGENASE (SDH, EC 1.1.1.14), especially in sink organs. SDH has also been found in non-Rosaceae species and here we show that the protein encoded by At5g51970 in *Arabidopsis thaliana* (L.) Heynh. possesses the molecular characteristics of an SDH. Using a green fluorescent protein-tagged version and anti-SDH antisera, we determined that SDH is cytosolically localized, consistent with bioinformatic predictions. We also show that *SDH* is widely expressed, and that SDH protein accumulates in both source and sink organs. In the presence of NAD+, recombinant SDH exhibited greatest oxidative activity with sorbitol, ribitol and xylitol as substrates; other sugar alcohols were oxidized to a lesser extent. Under standard growth conditions, three independent *sdh-* mutants developed as wild-type. Nevertheless, all three exhibited reduced dry weight and primary root length compared to wild-type when grown in the presence of sorbitol. Additionally, under short-day conditions, the mutants were more resistant to dehydration stress, as shown by a reduced loss of leaf water content when watering was withheld, and a greater survival rate on re-watering. This evidence suggests that limitations in the metabolism of sugar alcohols alter the growth of *Arabidopsis* and its response to drought.

1. Introduction

In higher plants, photo-assimilated carbon is translocated through the phloem as sucrose. However, some families utilize other sugars as the means of transporting assimilated carbon, such as raffinose or stachyose in Cucurbitaceae [1], or sugar alcohols. Sugar alcohols (also known as polyols) include cyclic forms, such as *myo*-inositol, and linear molecules including sorbitol, mannitol, xylitol and ribitol. Mannitol, can account for up to 60% of the phloem-translocated carbon in members of the Apiaceae, Rubiaceae and Oleaceae families [2]. However, in the phloem of Rosaceae and Plantaginaceae species, sorbitol (the reduced form of glucose, also known as glucitol) is the principal carbon form present [3]. The translocation of sorbitol also assists in the plants ability to withstand environmental stresses. For example, boron (B) is a micronutrient essential for efficient growth, particularly in plant cell wall formation [4]. The presence of sorbitol and mannitol also enables the redistribution of B from mature to younger organs *via* the phloem, meaning that polyol-translocating species suffer less from B-deficiency than those that translocate sucrose [5,6]. Additionally, sugar alcohols serve as osmoprotectants, and accumulate in plants subjected to a variety of abiotic stresses, including drought, cold and high salinity [7,8].

On entering fruits of some Rosaceae species, sorbitol levels rise dramatically during ripening, contributing to the sweetness of the fruit (*e.g.* sour cherry, *Prunus cerasus*; [9]). However, in other species little accumulation of sorbitol occurs, suggesting it is rapidly metabolized to other carbohydrate forms. Two enzymes have been identified in sorbitol conversion, NAD+-dependent SORBITOL DEHYDROGENASE (SDH, EC 1.1.1.14) and sorbitol oxidase, which oxidize the inert sugar alcohol to metabolically accessible

Abbreviations: NAD+, nicotinamide adenine dinucleotide; RDH, ribitol dehydrogenase; RWC, relative water content; SDH, sorbitol dehydrogenase; SGWC, soil gravimetric water content; XDH, xylitol dehydrogenase.

* Corresponding author at: Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile. Tel.: +56 2 9787263; fax: +56 2 2712983.
E-mail addresses: igneea@hotmail.com (M.F. Aguayo), dampuero@u.uchile.cl (D. Ampuero), patriciomandujano@gmail.com (P. Mandujano), tito.rps@gmail.com (R. Parada), rmunoz@network-chile.cl (R. Muñoz), madigal9@gmail.com (M. Gallart), taltabella@ub.edu (T. Altabella), ricabrer@uchile.cl (R. Cabrera), cstange@uchile.cl (C. Stange), mhandfor@uchile.cl (M. Handford).
[1] Present address: Centrovet, Avenida Los Cerrillos 602, Cerrillos, Santiago, Chile.

0168-9452/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.plantsci.2013.01.012

fructose and glucose, respectively. Sorbitol oxidase activity has been detected in sink tissues of apple (*Malus* × *domestica*; [10]) and peaches (*P. persica*; [11]), but no plant sorbitol oxidases have been cloned to date. SDH activity is high during the maturation of non-sorbitol-accumulating fruits, such as peaches [11,12], Japanese pears (*Pyrus pyrifolia*; [13]) and loquats (*Eriobotrya japonica*; [14]), and several cDNAs encoding apple SDH have been cloned [15–18]. Of these, *MdSDH2*, *3* and *4* are particularly highly expressed in sink tissues such as fruits, during the period of main sorbitol influx into these organs [16]. Heterologous expression in *Escherichia coli* demonstrated that MdSDH3 had the greatest activity of those apple SDHs tested, and converted sorbitol to fructose 4 times more efficiently than the reverse reaction [16]. A more extensive analysis of substrate specificity of SDH was undertaken using a purified pear form [13]. Whereas sorbitol was oxidized with highest efficiency, 5-carbon xylitol and ribitol were catalyzed with 76% and 14% efficiency, respectively, but mannitol oxidation was not detected.

SDH activity has also been identified in non-sorbitol translocating species including soybean (*Glycine max*, Fabaceae; [19]) and maize (*Zea mays*, Poaceae; [20]). As in the case of SDHs characterized from the Rosaceae family, maize SDH and a recombinant LeSDH from tomato (*Solanum lycopersicum*, Solanaceae) were also capable of oxidizing other sugar alcohols, albeit with lesser efficiency [20,21]. Nevertheless, the potential roles of SDH activity in non-sorbitol translocating species are unclear. SDH is not exclusive to the plant kingdom; indeed in animals, this enzyme forms part of the polyol pathway, a mechanism of shunting glucose to fructose, *via* sorbitol [22]. The crystal structures of human and rat SDH have been determined suggesting that the enzyme functions as a homotetramer [23,24] and incorporates a catalytic zinc ion. Human SDH, encoded by *SORD*, oxidizes several polyols including sorbitol, xylitol, ribitol and L-threitol [25]. Additionally, SDH encoded by *SOR1* is found in *Saccharomyces cerevisiae*. Sorbitol and xylitol, but not mannitol oxidation were detected for the yeast SDH [26].

In *Arabidopsis thaliana* (Brassicaceae), the most abundant form of phloem-translocated carbon is sucrose, along with much lower concentrations of raffinose [27]. Sorbitol is not present in the phloem under standard growth conditions and B is transported as boric acid (and not complexed with sorbitol) in *Arabidopsis* [28]. Nevertheless, metabolic profiling studies have detected the presence of several sugar alcohols in *Arabidopsis*. *myo*-Inositol and a range of linear sugar alcohols, including glycerol, erythritol, xylitol, ribitol, mannitol and sorbitol have been identified in *Arabidopsis* [29–33]. Bioinformatically, a potential SDH was identified in *Arabidopsis* (At5g51970; [21]), and in a recent study [34], *sdh*- mutants were shown to have a mild increase in sorbitol, and a substantial increase in ribitol levels after drought stress, as well as reduced SDH and ribitol dehydrogenase (RDH, EC 1.1.1.56) activities *in planta*.

Here, we further characterize *Arabidopsis* SDH, and show using *in vitro* and yeast expression systems, that SDH has a greater capability for oxidizing sorbitol, ribitol and xylitol than for oxidizing other sugar alcohols found in plants. Subcellular localization analysis indicates that SDH is present in the cytosol and *SDH* is widely expressed in *Arabidopsis*. In addition, three independent *sdh*- mutants present conditional growth defects, leading us to suggest that limitations in the metabolism of sorbitol alter the growth and development of *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

All lines of *Arabidopsis* (*A. thaliana* Heynh. ecotype Columbia) were maintained at 22 °C under a 16 h light/8 h dark regime at 6000–8000 lx, unless stated otherwise. For selection and genotyping, seeds of wild-type, mutant and transformed lines were surface sterilized and plated onto Murashige and Skoog [35] medium (1% Suc, pH 5.8) supplemented with kanamycin (50 μg mL^{-1}) or hygromycin (10 μg mL^{-1}). Phenotypic analyses of wild-type and mutants were performed after germinating seeds on MS, or MS supplemented with 30 mM sorbitol or sucrose on plates at an angle of 70°.

2.2. Cloning of SDH

SDH cDNA was amplified from *Arabidopsis* rosette leaf cDNA using Pfu polymerase (Fermentas) and primers SDH-BamHIF (5′-GGCAGGATCCAAATGGGGAAAGGAGGGATG-3′) and SDH-NotIR (5′-GGACGCGGCCGCGAGTTGAACATAACTTTCATG-3′). Both primers flank the coding region and the PCR product was cloned into pCR8 (Invitrogen) forming pCR8-SDH and then homologously recombined into the Gateway-compatible vector, pGWB5 [36] for the addition of a C-terminal GFP tag. To generate recombinant His-SDH *in vitro*, primers SDH-F (5′-ATGGGG AAAGGAGGGATGTCTCAAG-3′) and SDH-R (5′-TCAGAGATTGAACATAACTTTGATG-3′) and template pCR8-SDH were used, and the PCR product was cloned into pEXP5-NT/TOPO (Invitrogen). The putative promoter, composed of the 692 bp upstream of the *SDH* start codon and without including the coding sequence of the neighboring gene (At5g51960), was amplified from genomic DNA using primers SDH-BamHI5 (5′-GGACGGATCCTTCCTCTTTGTCTTTCCGG-3′) and SDH-NcoI3 (5′-GGACCCATGGAACTCTTCTCTGCTATGCTC-3′) and cloned into pCAMBIA1381 (http://www.cambia.org), producing promSDH::GUS in which the promoter sequence lies upstream of the *β-glucuronidase* reporter gene. For expression in yeast, *SDH* cDNA was ligated into BamHI/NotI-digested p426-GPD-myc-His [37] for the addition of a C-terminal myc-His tag, after amplification using primers SDH-BamHIy5′ (5′-GGACGGATCCATGGGGAA AGGAGGGTG-3′) and SDH-NotIy3′ (5′-GGCAGCGGCCGCAAGAGATTGAACATAACTTTG ATG-3′). All clones were verified by sequencing.

2.3. Expression of His-SDH and SDH-myc-His

His-SDH was expressed *in vitro* from the pEXP5-NT/TOPO vector using the Expressway Cell-Free expression system (Invitrogen) according to the manufacturer's instructions with minor modifications (1.5 μg of plasmid DNA per reaction; expression at 30 °C for 6 h). For purification, the *in vitro* reaction (250 μl) was resuspended in binding buffer (100 mM Tris–HCl pH 8.2, 500 mM NaCl, 10 mM imidazole, 10% glycerol) and loaded onto a His-Spin Protein Miniprep (Zymo-Research) and the column washed with 10 volumes of binding buffer containing 50 mM imidazole. Bound proteins were eluted with binding buffer containing 250 mM imidazole.

Yeast BY4743 cells were transformed cells with p426-GPD-myc-His or p426-GPD-SDH-myc-His and grown on YMM (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose and 0.07% amino acids mix 1 (100 mg adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan) and 0.045% mix 2 (50 mg of aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine). After growth at 28 °C ($OD_{600\,nm}$ 8), proteins were extracted from harvested cells using CelLyticY (Sigma) according to the manufacturer's instructions.

The recombinant proteins were detected by immunoblot analysis using monoclonal anti-His (Sigma; to detect His-SDH) or anti-c-Myc (A-14, Santa Cruz Biotechnology; to detect SDH-myc-His) antisera and SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific).

2.4. Substrate specificity analysis of SDH

Crude extracts containing His-SDH or SDH-myc-His were subjected to zymogram analysis in a native PAGE gel (12%, 6 h, 4 °C, 80 V) according to de Sousa et al. [38], using 68 mM sugar alcohol. As positive control, SDH from sheep liver (Sigma) was employed. In the case of His-SDH and SDH-myc-His, crude extracts of the *in vitro* reaction performed with the control vector supplied by the kit manufacturers, and yeast transformed with the empty vector (p426-GPD-myc-His) were used as negative controls, respectively.

Dehydrogenase activity was determined spectrophotometrically by measuring the rate of change in absorbance at 340 nm for NAD+ reduction at 25 °C, using a Biotek Synergy 2 spectrophotometer. Reactions were initiated by adding purified His-AtSDL (1.5 ng) to a standard reaction mixture containing 100 mM Tris–HCl pH 9, 50 mM sugar alcohol and 2 mM NAD+. In separate experiments, sorbitol and NAD+ concentrations were varied in order to determine the *K*m of both substrates.

2.5. Protein fractionation and immunoblotting

To determine the subcellular localization of native SDH, 1 g of rosette leaves was ground in protoplast buffer (400 mM sorbitol, 20 mM MES/KOH pH 5.7, 0.5 mM $CaCl_2$; [39]). The suspension was filtered (100 μm pore) and centrifuged (1000 × *g*, 20 min) forming pellet (P1) and supernatant (S1) fractions. S1 was centrifuged (10,000 × *g*, 30 min) forming P10 and S10, the latter of which was recentrifuged (100,000 × *g*, 90 min), resulting in P100 and S100 fractions. The three pellet fractions were resuspended (100 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1.5% Triton X-100, 0.1% SDS, 1 mM DTT, 0.1 mM PMSF), whereas S100 was precipitated with 20% trichloroacteic acid, centrifuged (10,000 × *g*, 15 min) and resuspended in 100 mM NaOH. To determine the presence of SDH in different organs, 100 mg of tissue was ground in protoplast buffer, filtered (100 μm pore), centrifuged (10,000 × *g*, 20 min) and the supernatant precipitated and resuspended as described above. Protein concentrations were determined according to the Bradford [40] method. Samples were fractionated by 12% SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. Polyclonal anti-SDH antiserum was raised in mice against recombinant GST-SDH purified from *E. coli* transformed with pGEX-5x-SDH, a vector which harbors *SDH* cDNA cloned between BamHI (5′) and NotI (3′) restriction sites of pGEX-5x (GE Healthcare). When using the anti-SDH antiserum, membranes were blocked for 1 h at 22 °C in TBS-T (20 mM Tris–HCl pH 7,5; 140 mM NaCl; 0.1% Tween-20) and 3% BSA, followed by incubation in anti-SDH antiserum (1:100 dilution) overnight at 4 °C in TBS-T/1% BSA. Antibody binding was detected using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific).

2.6. RNA isolation and quantitative expression analysis of SDH in Arabidopsis

Total RNA from roots and rosette leaves (25 d), and from stems, cauline leaves, flowers and siliques (8 weeks) was isolated using the Ultra Clean Plant RNA isolation kit (MOBIO Laboratories). RNA samples from dry seeds (12 weeks) were prepared using a TRIzol method (Invitrogen; [41]). RNA concentration and integrity were measured after DNase I (Fermentas) treatment with a NanoDrop 3300 Fluorospectrometer (ThermoScientific) and agarose gel electrophoresis under denaturing conditions. By use of a common oligo AP (5′-CGCCACGCGTCG ACTAGTACTTTTTTTTTTTTTTTT-3′) primer, 3 μg of DNAse I-treated total RNA were heated (70 °C, 5 min) and then subjected to a reverse transcription (RT) reaction using Improm-II reagents (Promega). Quantitative polymerase chain reactions (qPCR) were performed with an Mx3000P Real-Time PCR System (Stratagene, CA), using SensiMix SYBER Hi-Rox kit (Bioline) to monitor dsDNA synthesis. The following standard thermal profile was used for all qPCR reactions: 25 °C for 1 s, 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 s. Amplicon dissociation curves were recorded after cycle 40 by adding one cycle of 95 °C for 5 s, 25 °C for 1 s and a heating step from 55 °C to 95 °C with a ramp speed of 1.9 °C min^{-1}. Data were analyzed using MxPro system software (Stratagene). The expression of three reference genes, *UBI10* (At4g05320), *PP2A* (At1g13320) and *At4g26410* [42] was monitored and *At4g26410* was selected using NormFinder [43]. *SDH* transcript levels were normalized to the expression of *At4g26410* using the Δ*Ct* method [44] and relativized against the organ with lowest *Ct* value. The sequences of the primer pairs used were: *SDH* (5′-TGTGTCTTGAGTTCCTGACAAGTGGT-3′ and 5′-ATTGCTCCCACGAGCACTGGTTTCA-3′), *UBI10* (5′-GGCCTTGTATAATCCCTGATG AATAAG-3′ and 5′-AAAGAG ATAACAGGAACGGAAACATAGT-3′), *PP2A* (5′-TAACGTGGC CAAAATGATGC-3′ and 5′-GTTCTCCACAACCGCTTGGT-3′) and *At4g26410* (5′-GAGCTGAA GTGGCTTCCATGAC-3′ and 5′-GGTCCGACATACCCATGATCC-3′). All qPCR data represent the average of two independent biological pools of samples and three technical replicates.

2.7. Subcellular localization of SDH

Tobacco leaves (*Nicotiana tabacum*, cultivar Xanthi NN) were used for transient expression after *Agrobacterium tumefaciens*-mediated transformation (strain GV3101) with pGWB5-SDH-GFP by syringe infiltration using the method described in Handford et al. [45]. Infiltrations with *Agrobacterium* harboring pCAMBIA1302 (35S-GFP; http://www.cambia.org) were performed as a positive control. Lower epidermal peels of transformed leaves were analyzed 4 d after infection with a bacterial culture ($OD_{600\,nm}$ 0.6–0.8). All images were captured with 40× augmentation after excitation at 489 nm with an epifluorescence microscope (Olympus IX70) and processed with Scion Image.

2.8. Detection of GUS activity

Arabidopsis plants were transformed by the floral dip method [46] with promSDH::GUS. Transformed lines were selected on MS medium supplemented with hygromycin 10 μg mL^{-1} and resistant plants were transferred to hydroponic medium after 7 d. After 3 weeks of development, leaf tissue was removed to determine the presence of the transgene by PCR. T3 homozygous lines were analyzed histochemically for GUS activity throughout development (1–8 weeks), as described previously [47], except that tissues were vacuum infiltrated for 60 min at 85 kPa. Images were obtained with an Olympus MVX10 macro zoom fluorescence microscope equipped with a QImaging Micro Publisher 3.3 RTV camera and QCapture Pro 6.0 software.

2.9. sdh- mutant identification and molecular characterization

The *sdh*- mutants, *sdh1-1*, *sdh1-3* and *sdh1-4* were ordered from the Nottingham Arabidopsis Stock Center (NASC; SALK_020855, SALK_077335, and SALK_023456, respectively; [48]). The presence and position of the T-DNA insert in each *sdh*- line was determined by PCR-screening using the following gene- and T-DNA-specific primers shown in Fig. 5A: pSDH-F (5′-GACGGAT CCTTCCTCTTTGTCTTTCCGG-3′; primer 1), pSDH-R (5′-GGACCCATGGA ACTCTTCTCTG CTATGCTC-3′; primer 2), SDH-F (primer 3), SDH-R (primer 4) and LBb1.3 (5′-ATTTTGCCGA TTTCGGAAC-3′). Homozygous plants were identified by PCR following the method described in

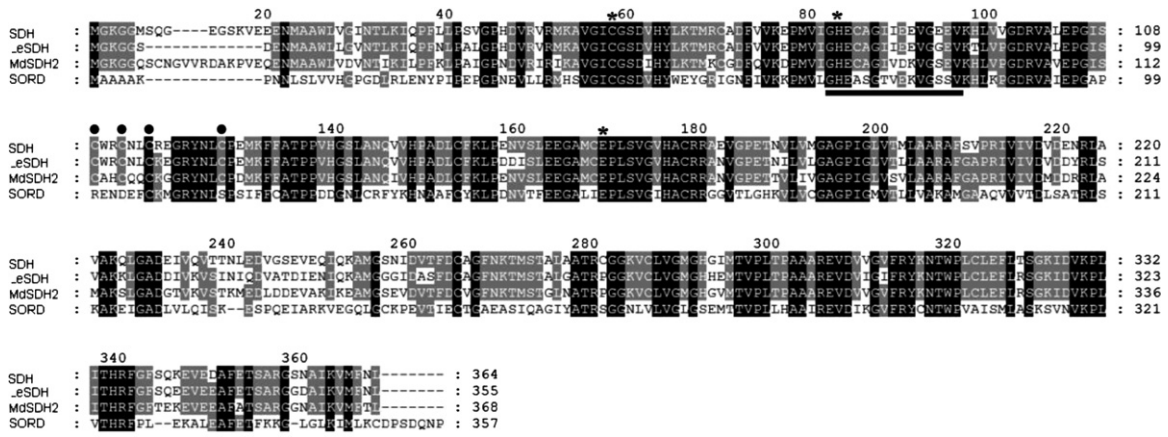

Fig. 1. Sequence analysis of *Arabidopsis* SDH. Alignment of SDH (GI: 15242240) with polyol dehydrogenases from *Solanum lycopersicum* (tomato; LeSDH, GI: 78183416), *Malus* × *domestica* (apple; MdSDH2, GI: 37932831) and *Homo sapiens* (human; SORD, GI: 156627571). Black or gray shading indicate identical amino acids conserved in all, or in three of the four species under comparison, respectively. Asterisks indicate conserved residues in the catalytic zinc-binding site, and circles indicate conserved residues in the structural zinc-binding site in the plant enzymes. The zinc-containing alcohol dehydrogenase signature is underlined.

http://signal.salk.edu/tdnaprimers.2.html. Gene expression levels were determined as described above using total RNA isolated from 8-d old seedlings, and proteins extracted from 10 d-old seedlings were subjected to immunoblot analysis.

2.10. Drought stress treatment

Experiments were performed as described previously [49]. Briefly, wild-type and *sdh*- mutants were sown in a mixture of soil and vermiculite (3:1 [v/v]) and after stratification, transferred to standard short-day conditions of 8 h/16 h light/dark cycles and fertilized with 0.5× Hoaglands's solution. Drought treatments were applied to 4-week-old plants by withholding further watering during 14 d. After this period of water deprivation, plants were re-watered as before. Drought tolerance was determined as the capacity of plants to survive after 14 d of recovery. The relative water content (RWC) and the soil gravimetric water content (SGWC) were calculated as described by Alcázar et al. [49].

3. Results

3.1. Molecular characteristics of Arabidopsis SDH

The predicted protein sequences of multi-specific SDHs from apple (MdSDH2, [16]), tomato (LeSDH, [21]) and human (SORD, [24]) were aligned with the gene product of At5g51970 (Fig. 1). The *Arabidopsis* SDH shares 77–83% amino acid identity with the plant enzymes and 42% with human SDH. Comparison of the *SDH* cDNA with the genomic sequence highlights the presence of 5 exons and the predicted protein produced has 364 amino acids and an estimated molecular weight of ~39 kDa. Interestingly, highly conserved cysteine residues involved in structural zinc binding site, as well as sequences of a zinc-containing alcohol dehydrogenase signature and a catalytic zinc-binding site, are present (Fig. 1; [12,21,50]). Taken together, these molecular characteristics are consistent with the product of At5g51970 being an SDH.

3.2. Enzymatic activity of Arabidopsis SDH

In order to determine the substrate specificity of SDH, an N-terminally tagged His fusion version was generated in an *in vitro* transcription and translation system. After induction of protein expression, a single protein of the expected molecular weight of His-SDH (~42 kDa) was detected using anti-His antisera (Fig. 2A). In a preliminary analysis of the specificity of SDH, we made use of zymogram assays [38]. Crude extracts containing recombinant His-SDH, as well as commercially available sheep SDH, were resolved in a native PAGE gel, which was then incubated in a sugar alcohol-containing solution before being subjected to an in-gel colorimetric assay for the indirect detection of NAD+ reduction. Using this system, activity of His-SDH with sorbitol was clearly detected; in addition His-SDH also oxidized five-carbon xylitol, but not erythritol (4C) or glycerol (3C; Fig. 2C).

The vector used for the production of His-SDH *in vitro* adds the epitope to the N-terminus of the protein. In order to discard the possibility that this epitope was altering the substrate preference of the enzyme, SDH was expressed heterologously in *S. cerevisiae* with a C-terminal myc-His tag. SDH-myc-His protein expression in yeast was detected using anti-myc antisera (~45 kDa, Fig. 2B). Crude extracts were subjected to zymogram analysis, and as in the case of His-SDH, SDH-myc-His was capable of oxidizing sorbitol and xylitol, but not erythritol and glycerol (Fig. 2C). It should be noted that the signals detected in the negative control lane, corresponding to crude extracts isolated from yeast transformed with the empty vector control, probably correspond to the activity of multi-specific endogenous alcohol dehydrogenases, including SOR1 [26].

These qualitative results were then used as the basis to determine quantitatively the kinetic parameters of SDH for its substrates. The product of the *in vitro* transcription and translation system was purified with a Ni-affinity column, and a single protein of the expected molecular weight of His-SDH (~42 kDa) was detected using anti-His antisera (Supplementary Fig. 1). The purified protein was then used in spectrophotometric assays and specifically reduced NAD+, but not NADP+, in the presence of sorbitol, confirming that it is a NAD+-dependent SDH. Using the fit to a hyperbolic function at different substrate concentrations, the *K*m values for sorbitol and NAD+ were calculated to be 1.2 mM and 0.07 mM, respectively (Fig. 3A and B). The specific activity of the enzyme was determined with a range of *Arabidopsis* sugar alcohols, and was determined to be greatest with sorbitol (Fig. 3C). If the specific activity with this sugar alcohol is normalized to 100%, then ribitol (98%) and xylitol (80%) were also oxidized at high rates, whereas the relative activities with other sugar alcohols (arabitol 59%, mannitol 32%, maltitol 13%, lactitol 14% and erythritol 7%) were lower, which validate the qualitative results obtained using crude extracts containing His-SDH and SDH-myc-His in zymogram assays (Fig. 2C). Thus sugar alcohols with the same configuration at C-2 (S) are preferentially oxidized by SDH (Fig. 3D).

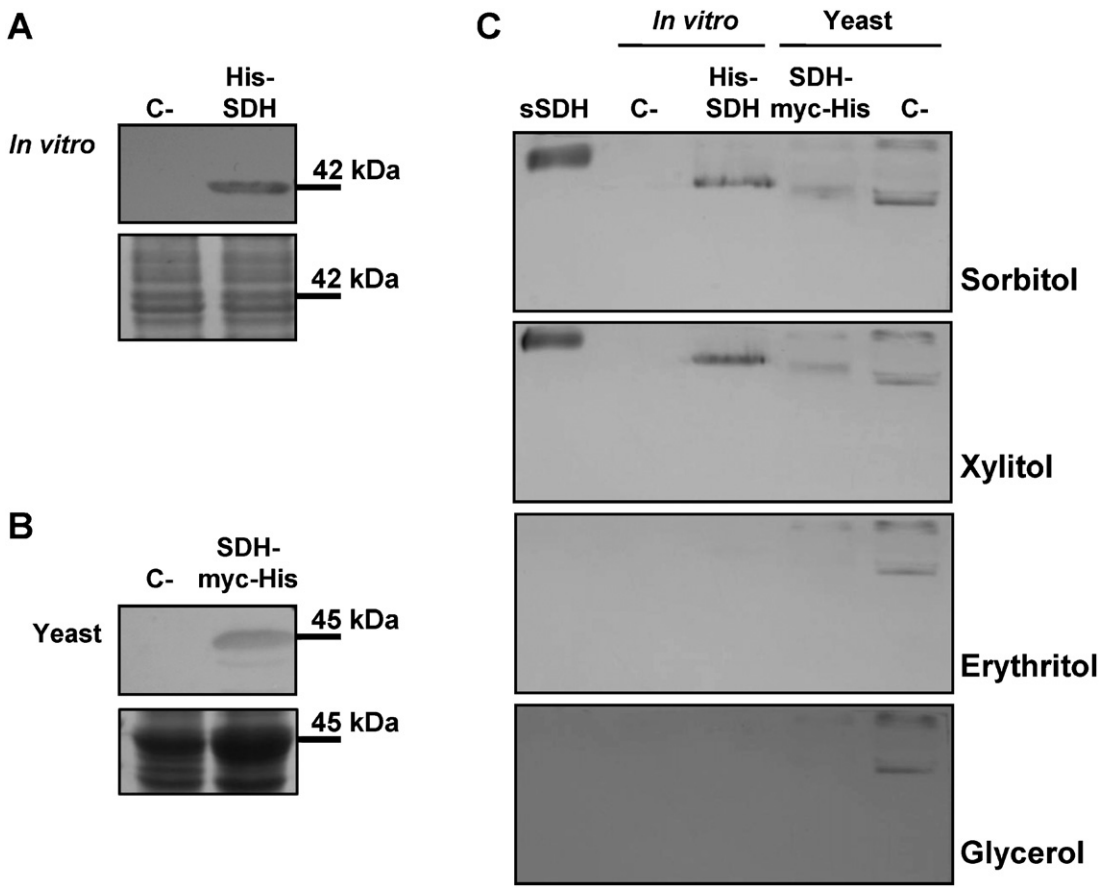

Fig. 2. Expression and zymogram analysis of recombinant SDH. Upper panels: immunoblot analysis of crude extracts containing recombinant SDH produced *in vitro* ((A) His-SDH; anti-His antisera) and in yeast ((B) SDH-myc-His; anti-myc antisera). C−, negative controls corresponding to crude extracts of the *in vitro* reaction performed with the control vector supplied by the kit manufacturers (in A), and yeast transformed with the empty vector (in B). Lower panels: respective Coomassie stained gels. (C) Zymogram analysis in native gels using crude extracts containing recombinant SDH produced *in vitro* and in yeast. sSDH; commercial sheep liver SDH used as a positive control. All gels were incubated with their respective substrate for 30 min at 37 °C, and then treated identically to a colorimetric visualization assay.

3.3. Expression pattern of Arabidopsis SDH

In order to examine the expression pattern of *Arabidopsis SDH*, quantitative real-time PCR was employed, demonstrating that transcripts accumulate in all organs tested and are most abundant in dry seeds, and cauline and rosette leaves (Fig. 4A). To analyze these findings at a greater resolution, *Arabidopsis* lines were stably-transformed with the *SDH* promoter fused to the *β-glucuronidase* reporter gene (promSDH::GUS). Of the 17 lines obtained, five were evaluated histochemically throughout development. The results confirm that *SDH* is expressed ubiquitously in *Arabidopsis*, and in all stages of development. However, GUS activity was not detected in all cell types. For example, in flowers, no activity was detected in the center of the style, in stigmas or in pollen sacs, whereas in roots, only the vasculature and columella cells were stained (Fig. 4B–I). The universal expression of SDH in this specie was also determined at the protein level, using mouse polyclonal antisera raised against recombinant *Arabidopsis* SDH fused to GST. Similar levels of a single protein of the expected size of endogenous SDH (∼39 kDa) were detected in all organs analyzed (Fig. 4J). We confirmed the specificity of the antisera generated, because no protein of the same size was detected in three independent T-DNA insertion *sdh1-* mutants (see below; Fig. 6C).

3.4. Subcellular localization of Arabidopsis SDH

Computer-based predictions of the SDH amino acid sequence indicate that the protein lacks predicted hydrophobic transmembrane domains and could be located in the cytosol, or possibly in the chloroplast (http://suba.plantenergy.uwa.edu.au/; [51]). In order to determine experimentally the subcellular localization of the protein, GFP was fused in frame to the C-terminus of SDH. SDH-GFP was then transiently expressed by *Agrobacterium*-mediated infiltration of tobacco (*N. tabacum*) leaves, using soluble GFP as a control. As reported previously, soluble GFP was detected in the cytosol 4 d post-infiltration [52]. On performing the same experiment with SDH-GFP, the resulting pattern of fluorescence around the border of the cells suggests that SDH-GFP is a cytosolic protein (Fig. 5B). Despite viewing cells after different numbers of days post-infiltration, a punctuate pattern of fluorescence coinciding with chlorophyll auto-fluorescence was not observed. A cytosolic localization for the proteins was confirmed using the anti-SDH antisera. Given that endogenous SDH was detected in *Arabidopsis* rosette leaves (Fig. 4J), these organs were harvested and subjected to subcellular fractionation. After this process, a protein of the expected size of native SDH (∼39 kDa) was visible only in the 100,000 *g* supernatant fraction, consistent with the protein being located in the cytosol (Fig. 5C).

3.5. sdh- mutant identification and molecular characterization

Given that recombinant SDH is capable of oxidizing several sugar alcohols (Figs. 2 and 3), we decided to examine the effect of abolishing *SDH* expression *in planta*. Thus, we identified and characterized three *SDH* T-DNA insertion lines in the Columbia (Col-0) ecotype: the SALK lines 020855 (designated *sdh1-1* by [34]),

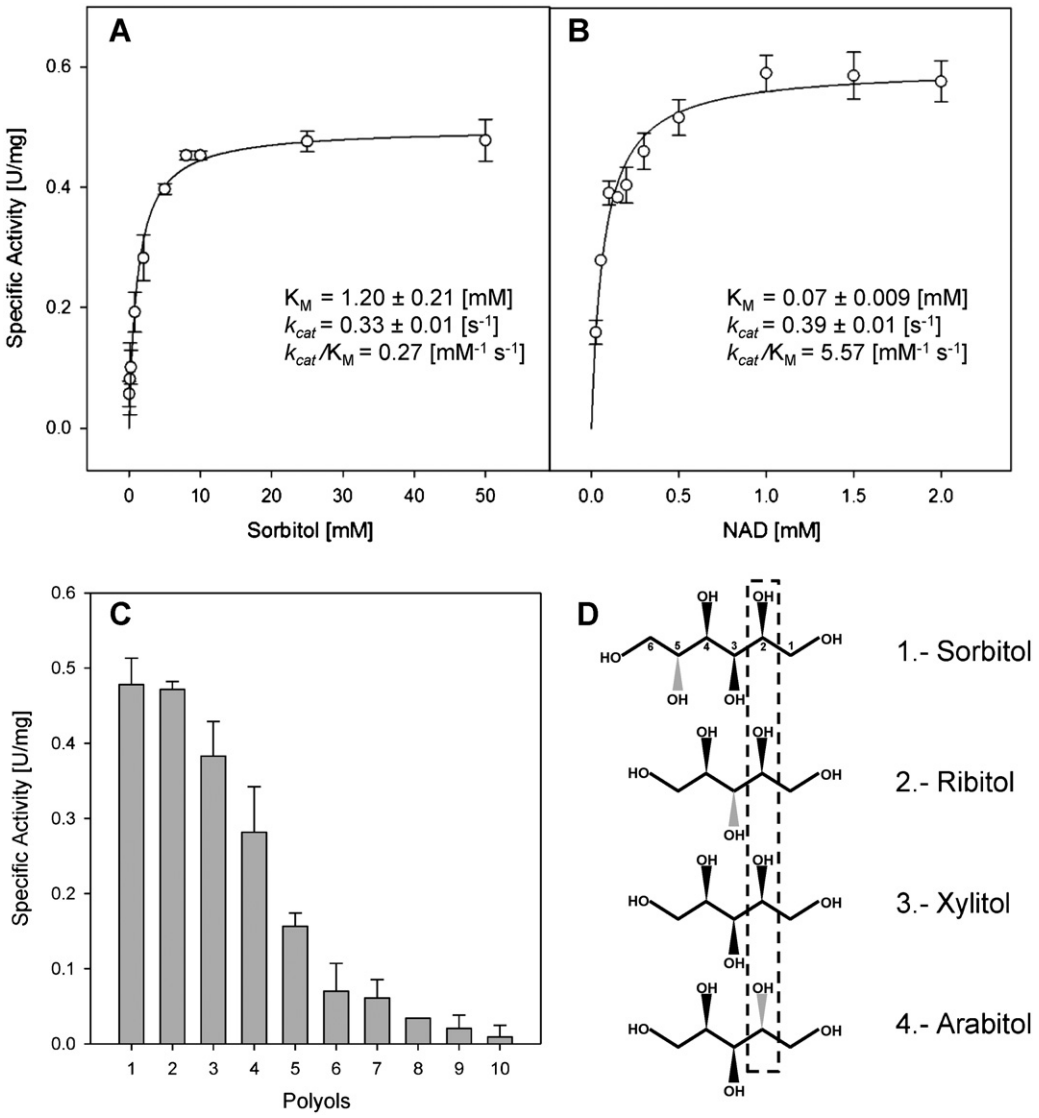

Fig. 3. Kinetic properties of recombinant His-SDH. Initial reaction rates were obtained with sorbitol (A) or NAD+ (B) as the variable substrate, whilst the co-substrate concentration was held saturating at 2 mM NAD+ and 50 mM sorbitol, respectively. The continuous lines represent the fit to a hyperbolic function from which *K*m and *k*cat values were estimated. (C) Comparison of the performance of His-SDH for the oxidation of eight different polyols ((1) sorbitol, (2) ribitol, (3) xylitol, (4) arabitol, (5) mannitol, (6) lactitol, (7) maltitol and (8) erythritol) and two sugars ((9) glucose and (10) fructose). The initial concentrations of NAD+ and polyol/sugar substrate were 2 and 50 mM, respectively. The stereochemical structure of those polyols showing more than 50% of the activity observed with sorbitol is shown in panel (D). The carbon (C-2) that becomes oxidized is boxed (dashed line). In (A), (B) and (C), values are the means ± SD of three independent determinations.

077335 (*sdh1-3*) and 023456 (*sdh1-4*). For each line, we isolated a homozygous mutant and PCR genotyping revealed that all mutant lines carry single T-DNA insertions. By sequencing the PCR products generated by the gene- and T-DNA-specific primer pairs, we determined that the T-DNA insertions were 250 bp upstream of the start codon in *sdh1-3* (5′UTR), and 1280 bp (exon 3) and 1688 bp (exon 5) downstream of the start codon in *sdh1-1* and *sdh1-4*, respectively, thus confirming the location of the T-DNA insert in *sdh1-1* ([34]; Fig. 6A). qRT-PCR analyses were employed to amplify a fragment located 3′ of all the insertions to determine the *SDH* mRNA expression levels in the mutants. The results indicate that transcript levels are severely reduced (<3% of wild-type levels) in all three lines, with the exon 5 insertion being effectively a knock-out line (*sdh1-4*; Fig. 6B). Furthermore, no SDH protein was detected in the three *sdh*- mutants with the anti-SDH antisera (Fig. 6C). In conclusion, the genetic and molecular analyses of each mutant line indicate the presence of a single T-DNA insertion in different exons of *SDH*, which severely diminish transcript and protein accumulation.

At the phenotypic level, the macroscopic growth and development of all *sdh*- mutants was indistinguishable from wild-type after germination and 15 d of growth in long-day conditions on MS alone or MS supplemented with 30 mM sucrose, in terms of both shoot dry weight and primary root length (Fig. 7A and B, white and black bars, respectively). Given that sorbitol is one of the substrates for SDH (Figs. 2 and 3), plants were grown on the same media supplemented with 30 mM of this sugar alcohol. As expected for such a low concentration of sorbitol [53], 30 mM of this sugar alcohol did not inhibit growth of wild-type plants (Fig. 7A and B, gray bars). However, these parameters fell significantly when the three lines without accumulation of SDH were propagated on the same medium. Specifically, dry weight was reduced by 30–37%, and root length by 26–30%, in the *sdh*- lines compared to wild-type (Fig. 7A and B). Together, these results demonstrate that the mutants have

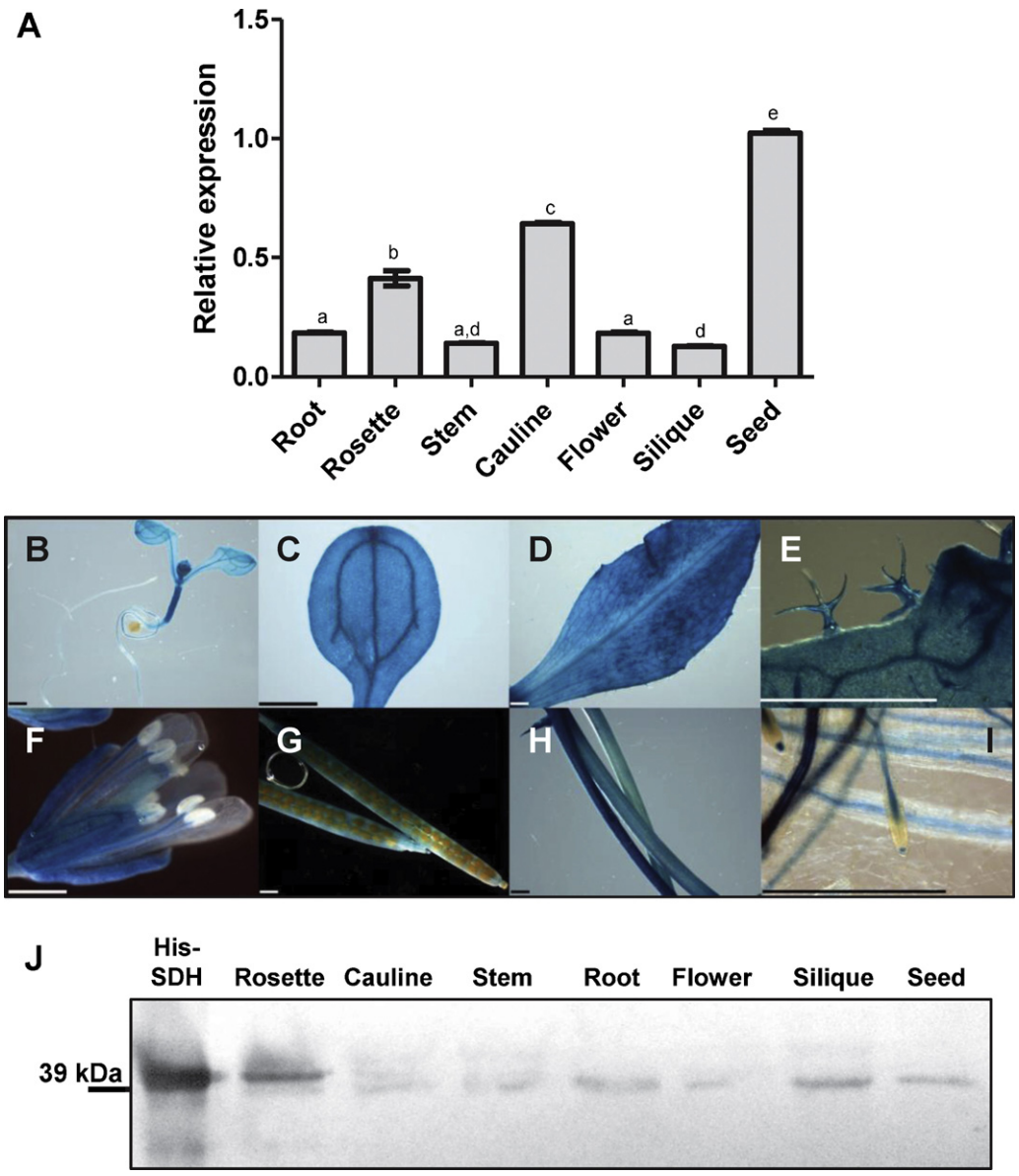

Fig. 4. Expression of SDH in *Arabidopsis*. (A) qRT-PCR analysis. SDH mRNA levels were determined by real-time quantitative RT-PCR, using total RNA from roots and rosette leaves (25 d), from stems, cauline leaves, flowers and siliques (8 weeks) and from dry seeds (12 weeks). The data were normalized using *At4g26410* accumulation as a control [42]. Different letters indicate significant differences for a specific organ as determined by means of one-way ANOVA and Bonferroni post-test ($p < 0.05$); SE bars are shown ($n = 3$). (B–J) GUS analysis. Samples were taken from plants stably-transformed with promSDH::GUS. 7-d seedling (B) and cotyledon (C), and rosette leaf (D) and trichomes (E), flowers (F), siliques (G), stems (H), and lateral roots (I) of 7-week old plants. Scale bar: 1 mm (J), accumulation of SDH. Proteins were extracted from *Arabidopsis* organs and subjected to immunoblot analysis using the anti-SDH antiserum. His-SDH; recombinant His-SDH used as positive control.

an impaired ability to grow on sorbitol as sole exogenous carbon source.

sdh- mutants were then tested for their drought tolerance and compared to wild-type plants. These experiments were carried out in soil-grown plants under environmentally regulated short-day conditions. Four-week-old lines were exposed to water deprivation by withholding water for 14 d. After this period, wild-type showed symptoms of dehydration, especially in older leaves (Fig. 8A). On the other hand, enhanced plant performance under drought stress was evident in all three *sdh*- mutant lines (Fig. 8A). To quantify the extent of the tolerance, the number of plants that resumed growth after 14 d of recovery by re-watering was determined, and the values were expressed as survival percentage. In three independent experiments with at least 22 plants per line, plants lacking expression of *SDH* were significantly more tolerant to drought than wild-type plants (Fig. 8B). Specifically, survival rates in the mutant lines were approximately 80%, compared to just 43% in wild-type plants. These results are thus consistent with a reduced ability to degrade compatible solutes, such as sorbitol, in *sdh*- mutants. To ensure that all lines were exposed to the same degree of water deprivation, the soil gravimetric water content (SGWC), a parameter to assess the amount of water in soil, was calculated. The decreasing values of SGWC during the course of the experiment did not differ significantly among groups (wild-type and mutant lines; Fig. 8C), indicating a similar water loss rate from soil through transpiration and/or evaporation in all plants, suggesting that the imposed drought stress between lines and wild-type was comparable. Additionally, the rate of water loss was determined during drought stress. The three *sdh*- mutant lines, which showed the highest survival rates (Fig. 8B), exhibited significant differences in relative water content (RWC) compared to wild-type plants after water deprivation (Fig. 8D). When water uptake by roots is balanced

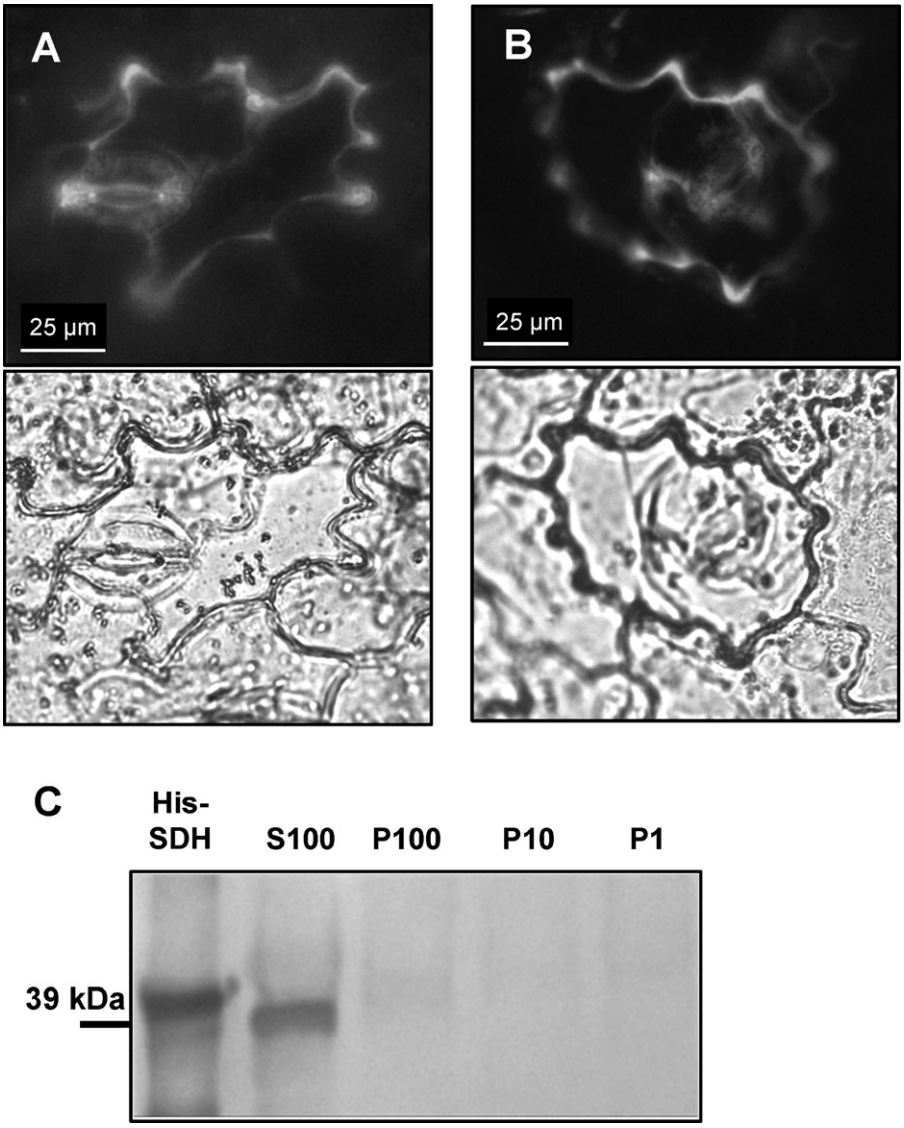

Fig. 5. Subcellular localization of SDH. SDH-GFP was transformed into tobacco leaves by *Agrobacterium*-mediated syringe infiltration and samples observed after 4 d. Upper panels: distribution of GFP expressed from pCAMBIA1302 (A) and SDH-GFP (B). The internal fluorescence is due to the presence of guard cells, as observed in the respective bright field images (lower panels). (C) *Arabidopsis* rosette leaves were subcellularly fractionated using differential-speed centrifugation, and subjected to immunoblot analysis using the anti-SDH antisera. His-SDH; recombinant His-SDH used as positive control. P1, P10 and P100; 1000 × *g*, 10,000 × *g* and 100,000 × *g* pellets, respectively. S100; 100,000 × *g* supernatant.

with water loss by transpiration in leaves, the RWC is typically around 0.9. During the first 11 d of drought stress, the RWC was similar in all plants. Nevertheless, after 14 d of imposed water-deficit stress, the RWC of wild-type plants decreased markedly to a value of 0.54, whereas that of the three lines lacking *SDH* expression, fell marginally to values ranging from 0.76 to 0.82 (Fig. 8D).

4. Discussion

SDH from *Arabidopsis*, encoded by At5g51970, possesses the molecular characteristics of SDHs from both plants and animals, with conserved catalytic and structural zinc-binding domains. As observed for other plant SDHs, the SDH from *Arabidopsis* was capable of oxidizing a range of sugar alcohols, and the *K*m of this for sorbitol is similar to that reported for the tomato LeSDH [21]. The purified enzyme possessed almost identical specific activities with sorbitol and ribitol, supporting the suggestion that SDH is also an RDH in this species [34]. Nevertheless, in plant extracts, RDH activity was approximately one-third of SDH activity [34], implying that potentially other sugar alcohol dehydrogenases are present in this species. The two most similar sequences to SDH in *Arabidopsis* are At5g42250 and At5g63620, which share 25% and 26% amino acid identity to At5g51970, respectively. Both possess the same potential zinc-binding domains as SDH and so may function as sugar alcohol dehydrogenases in this species. However, the phenotypic defects of *sdh*- mutants grown on sorbitol-containing media or subjected to drought stress in this study and in [34] indicate that SDH is not functionally redundant with these two paralogues. Alternatively, other potential sugar alcohol dehydrogenases may be present.

Of the sugar alcohols tested in enzyme assays, those oxidized preferentially by recombinant *Arabidopsis* SDH were sorbitol (100%), ribitol (98%) and xylitol (80%). Xylitol is reduced to xylulose by xylitol dehydrogenase (XDH, E.C. 1.1.1.9), and a gene encoding this enzyme has been cloned from *S. cerevisiae* [54]. The protein sequence with highest identity to yeast XDH in *Arabidopsis* is SDH (35% identity). For these reasons, we propose that At5g51970 is also an XDH; however even though xylitol transporters AtPMT1

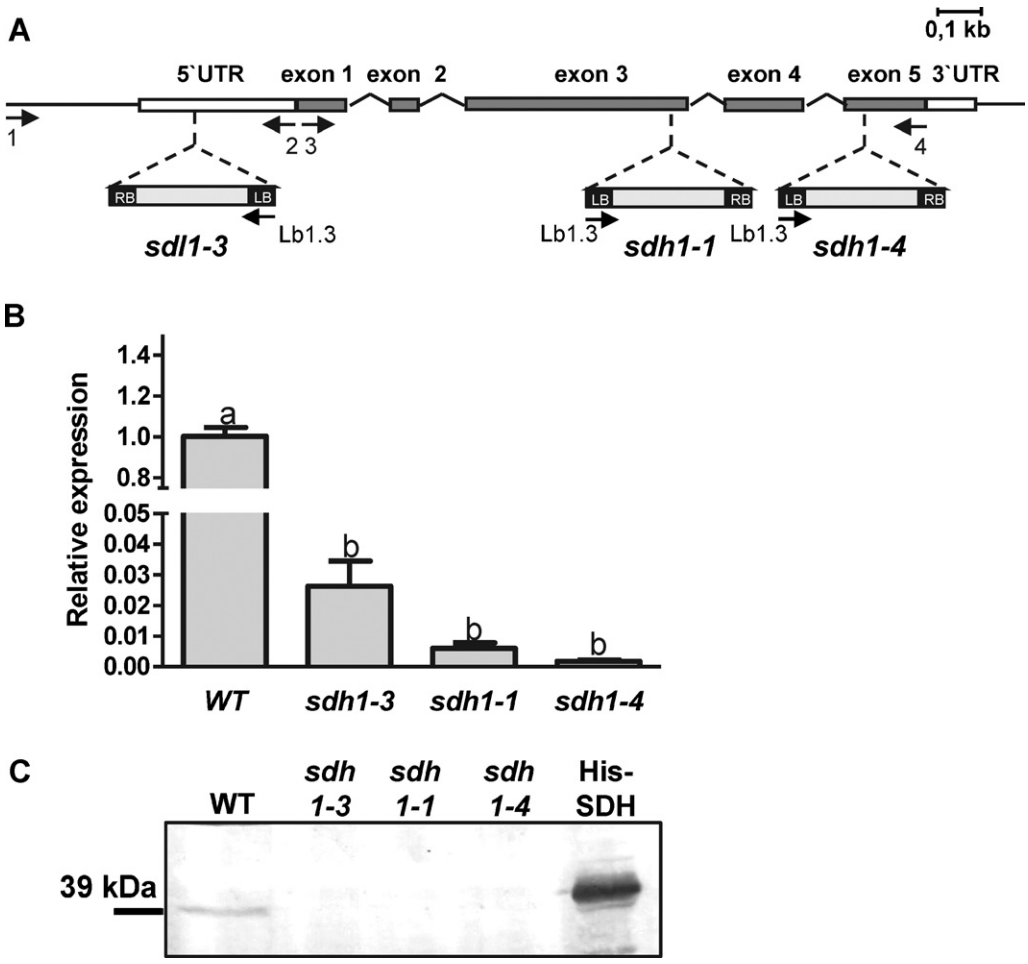

Fig. 6. Molecular characterization of *sdh1-* mutants. (A) Schematic representation of the positions of T-DNA insertions in the *sdl1-1*, *sdl1-3* and *sdl1-4* mutant lines. The names and locations of the PCR primers used for genotyping are shown. (B) qRT-PCR analysis of SDH expression in 8 d-old wild-type (WT) and homozygous *sdh1-* mutants. Values are the means ± SE of three determinations using *At4g26410* expression as a normalizer [42] and shown relative to expression levels in WT plants. Different letters indicate significant differences between expression levels as determined by means of one-way ANOVA and Bonferroni post-test ($p < 0.05$). (C) Accumulation of SDH. Proteins were extracted from *Arabidopsis* seedlings (10 d) and subjected to immunoblot analysis using the anti-SDH antiserum. His-SDH; recombinant His-SDH used as positive control.

and AtPMT2 are present in *Arabidopsis*, the physiological role of this sugar alcohol is unclear [55]. SDH has also been reported to be a RDH in *Arabidopsis* [34]. Ribitol, when converted by RDH is oxidized at C-2 to ribulose, similar to the oxidation of sorbitol or xylitol at C-2 to fructose or xylulose, respectively (www.genome.jp/kegg/pathway). Structurally, ribitol and xylitol both possess one C-atom less than sorbitol, but all three sugar alcohols have the same S and R configurations at C-2 (S) and C-4 (R; Fig. 3D). Molecules with different configurations at these two C-atoms were oxidized at a lower rate; L-arabitol (C-2 (S), C-4 (S); 59%) and D-mannitol (C-2 (R), C-4 (R); 32%), suggesting that this configuration is key for optimal catalytic activity [13]. Homology modeling and molecular dynamic studies of the *Arabidopsis* enzyme could help to identify the key amino acid residues involved in substrate binding and catalysis, and may provide an explanation for the preference of the C-2 (S) and C-4 (R) configuration. Ribitol and xylitol have been shown to be substrates of several plant SDHs. For example, maize SDH oxidizes ribitol and xylitol with 73% and 91% of the efficiency of sorbitol oxidation, respectively [20], and tomato LeSDH also catalyze ribitol and xylitol oxidation (60% and 29%, respectively [21]). Interestingly however, the SDHs characterized from Rosaceae species have a significantly lower ability to metabolize ribitol compared to xylitol. Apple SDH harbors between 7% and 12% activity with ribitol, yet 40–84% with xylitol compared to sorbitol [56,57], whilst pear SDH oxidizes ribitol and xylitol at 14% and 77% of the levels of sorbitol oxidation, respectively [13]. Recently, a NAD+-SDH from plum (*P. salicina*) has been characterized, and its activity follows the same trend (ribitol, 15%; xylitol, 63%; [58]). Thus it appears that the SDHs from non-Rosaceae species have a greater relative activity with ribitol than their Rosaceae counterparts, which may be reflected by subtle changes in the configuration of amino acids in the active sites of these enzymes. Taking into consideration that the specificity changes toward sorbitol, ribitol and xylitol are determined by differences in the amino acid sequences between these orthologous enzymes, it will be helpful to analyze the patterns of sequence conservation that could be related to the discrimination between the R and S configurations of the substrates. The comparison of the relative activities with different substrates (Fig. 3C) is a first step to understand the polyol specificity of SDH. A deeper understanding will involve a comparison of the *K*m and *k*cat, in order to reveal how these substrates are used over a broader concentration range. In fact, the ratio of the catalytic efficiencies (*k*cat/*K*m) for two alternative substrates is a good estimator of the relative rates in which they will be consumed by an enzyme, if they are present at equimolar concentrations [59].

Immunohistochemical analysis has demonstrated that in apples, SDH is present in both sink and source organs, and is localized in the cytosol, chloroplasts and vacuoles [18]. In this species, SDH is encoded by a large gene family composed of as many as 17

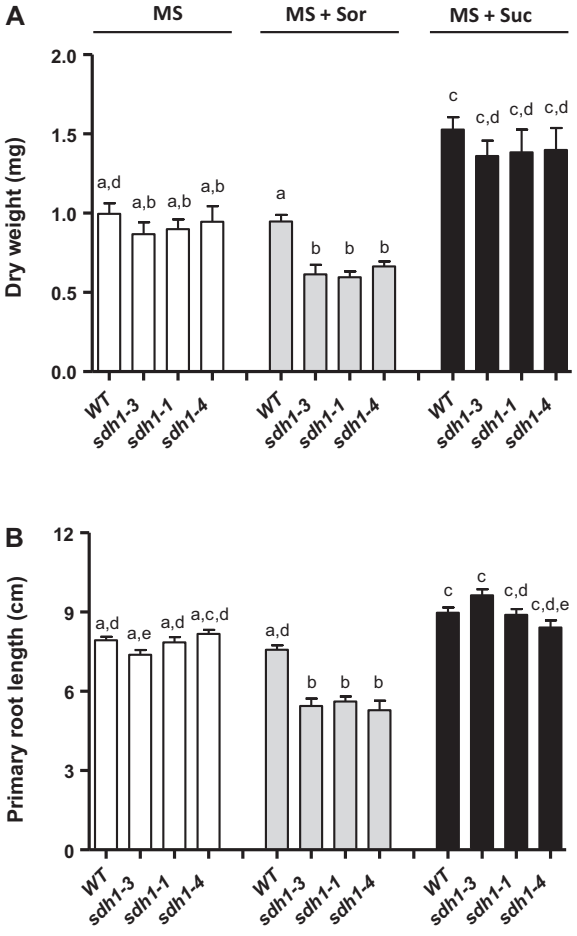

Fig. 7. Growth phenotypes of *sdh*- lines compared to wild type. Wild type (WT) and *sdh*- mutant lines were grown *in vitro* on MS (white bars), MS + 30 mM sorbitol (gray bars) and MS + 30 mM sucrose (black bars) media. After 15 d, shoot dry weight (A) and primary root length (B) were measured. Values shown are means ± SE (dry weight, $n = 13–44$; root length, $n = 13–60$). Different letters indicate significant differences between values of the same growth condition, as determined by means of two-way ANOVA and Bonferroni post-test ($p < 0.05$).

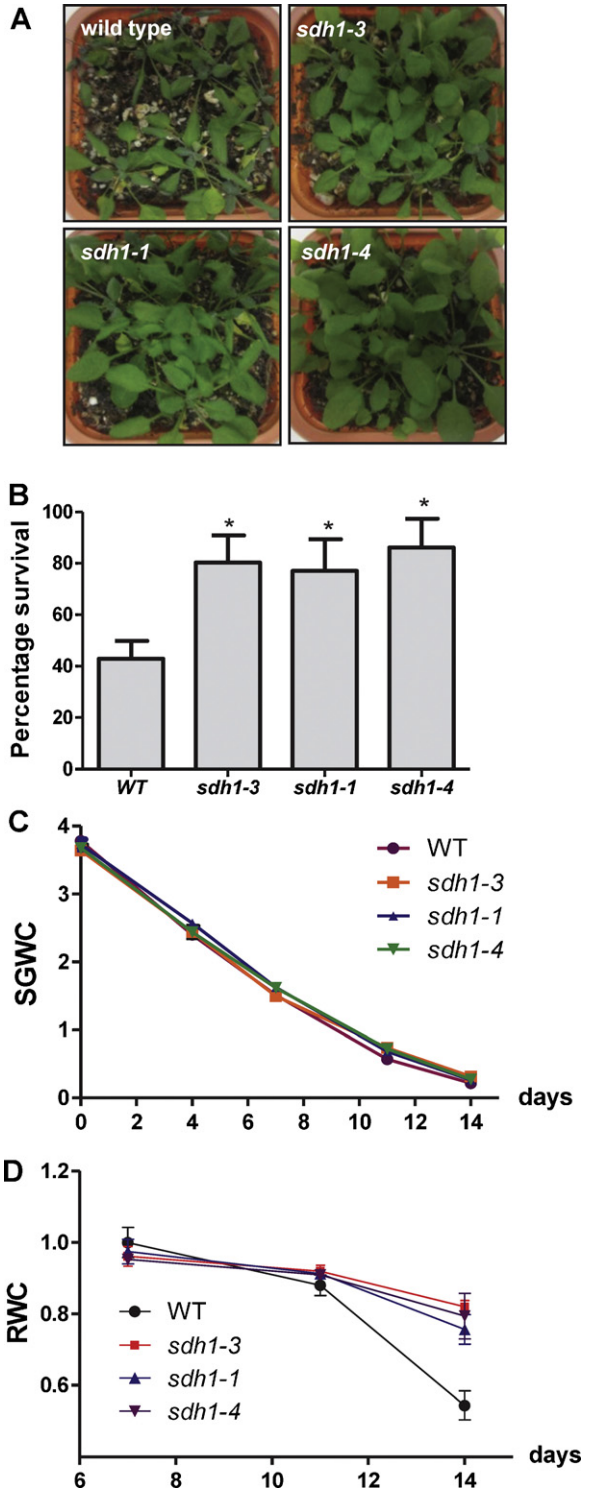

Fig. 8. Drought resistance phenotypes of *sdh*- lines compared to wild type. (A) Phenotype of 4-week-old plants grown under short-day conditions and dehydrated during 14 d. (B) Survival percentages of *sdh*- lines and wild-type (WT) plants after rehydration. (C) Soil gravimetric water content (SGWC) measured in the different pots containing *sdh*- lines and WT plants. (D) Relative water content (RWC) of *sdh*- lines and wild-type plants. Increased values of RWC after dehydration correlate with enhanced drought tolerance. Values shown are means ± SE of three independent experiments, each composed of 22–36 plants per line. Asterisks (*) indicate significant differences in survival with respect to WT plants ($p < 0.05$, *t*-test).

different genes [60] so potentially different gene products could be directed to different subcellular compartments. Using two different methods, we showed that *Arabidopsis* SDH is localized in the cytosol, results which are supported experimentally by an analysis of the *Arabidopsis* cytosolic proteome in which SDH was identified by mass spectrometry in this compartment [61]. The enzymes responsible for the synthesis of sorbitol in other plant species from glucose 6-phosphate (by ALDOSE 6-PHOSPHATE REDUCTASE, A6PR; EC 1.1.1.200) or glucose (by ALDOSE REDUCTASE, AR; EC 1.1.1.21) [62,63], have yet to be identified in *Arabidopsis*, although evidence from apples, indicates that A6PR is also present predominantly in the cytosol [64].

SDH transcripts were differentially-expressed in all organs tested, although the relatively constant levels of SDH protein present in the same organs likely reflect differences in transcript stability, translation rates and/or protein stability, among other factors. The highest expression levels were observed in dry seeds, findings consistent with those deposited in the *Arabidopsis* eFP Browser (http://bar.utoronto.ca; [65]). Unlike in other organs, in seeds SDH may act in the conversion of fructose to sorbitol with the concomitant production of NAD+ in order to maintain redox balance, as has been suggested for the activity of this enzyme in maize kernels [38].

Additionally, we identified and fused the *SDH* promoter to the *GUS* reporter gene and showed that the expression of this gene is essentially universal under standard conditions. Unlike the SDHs analyzed from Rosaceae species, which tend to be more highly-expressed in source organs [16], tomato *LeSDH* transcripts were detected in both source and sink organs, suggesting that this enzyme may play a different role in non-Rosaceae species [21]. *Arabidopsis* SDH transcription and activity were induced when plants were supplied with exogenous sorbitol [34]. Therefore, specific truncations of the promoter used in this work fused to GUS would be useful tools to identify potential *cis*-regulatory elements involved in the induction of expression of *SDH* by sorbitol, and other factors.

The three independent *sdh*- mutants, with <3% expression of *SDH*, all showed the same conditional phenotypic defects. On MS plates with and without 30 mM sorbitol, wild-type plants grew similarly, with no differences in dry weight and primary root length observable after 15 d under long-day conditions. Thus, it appears that once autotrophic, which typically occurs 48 h after germination [66], the presence of SDH in wild-type *Arabidopsis* does not affect the parameters measured, although it would be informative to examine their growth and development prior to this time point, to determine whether the ability to oxidize exogenously-supplied sorbitol is initially beneficial. As previously demonstrated [53], *Arabidopsis* does not usually show inhibitory effects of sorbitol until a concentration of over 300 mM. However, the *sdh*- mutants displayed a significant reduction in dry weight (by an average of 33%) and primary root length (28%) when grown on media supplemented with 30 mM sorbitol. This finding could indicate that, because the mutants are unable to convert exogenous sorbitol intracellularly, they suffer greater osmotic stress than wild-type plants due to an excessive accumulation of this compatible solute, resulting in reduced growth. The same phenomenon has been observed in tobacco plants over-expressing apple A6PR. When sorbitol levels were below ~5 μmol/g fresh weight, the transgenic tobaccos were phenotypically indistinguishable from wild-type [67,68]. Nevertheless, when this threshold was surpassed in the transformed lines, tobaccos suffered lower growth rates and a diminished rooting capability [68]. These, and similar studies in Japanese persimmon (*Diospyros kaki*; [69,70]) are in agreement with others which have shown that threshold levels of another compatible solute, the polyamine putrescine, exist in *Arabidopsis* [49]. The exogenously supplied sorbitol may enter *Arabidopsis* cells *via* multi-specific plasma membrane-localized H^+-symporters of the Polyol/Monosaccharide Transporter family, such as AtPMT1 and 2 [55] and AtPMT5 [71,72].

The *sdh*- mutants were also significantly more resistant to drought stress than wild-type, reflected by a higher survival percentage and an increased RWC in those lines lacking SDH accumulation. The greater RWC suggests that the higher survival rate is due to increases in compatible solutes in the mutants that help water retention in the leaves, and is likely a result of their inability to remove sugar alcohols such as sorbitol and ribitol, whose levels rise in drought stress [34]. Unlike all other experiments performed here, the drought stress assays were performed under short-day conditions in order to maintain vegetative growth of all lines for the 8-week duration of the study. This is important, as the switch to the reproductive growth phase has a profound effect on plant metabolism and could have altered the response of the mutants to this stress. Indeed, Nosarzewski et al. [34] showed that the *sdh1-1* mutant, which is common to both studies, was less resistant to dehydration stress when grown under long-day conditions during which the inflorescences were periodically removed. In long day conditions, it was postulated that the greater susceptibility of the mutants to drought stress was due to the levels of sorbitol, and especially ribitol, exceeding a critical threshold, as mentioned above [34]. It is interesting to note that in wild-type seedlings of the *Ler* ecotype, the average expression of *SDH* during a 24-h period is at least 30% greater in short days compared to long days, and that under both conditions, expression peaks in the dark period (65% higher expression levels in short days than in long days [73]). Therefore, it would be informative to examine whether day length in wild-type plants alters their susceptibility to dehydration stress, given that there is greater overall expression of *SDH* in short day conditions which could lead to a reduction in the concentration of sugar alcohol compatible solute levels.

Acknowledgements

We would like to thank Dr María Rosa Bono and Dr Leonardo Vargas for the production of the anti-SDH antisera, NASC Nottingham Arabidopsis Stock Center (UK) for supplying the *sdh1-1*, *sdh1-2* and *sdh1-3* seeds and Fabián Morales for assisting with their genotyping. This work was financed by Fondecyt 1100129 (M.H.) and 1121170 (R.C.), and partially supported by the U-REDES program 2012 of the VID, University of Chile. M.F.A. and D.A. were supported by Conicyt Beca de Magister Nacional grants 22110701 and 22100522, respectively. M.G. and T.A. were supported by the Spanish Government (CSD2007-00036 "Center for Research in Agrigenomics") and the Comissionat per Universitats i Recerca (Generalitat de Catalunya, SGR2009-1060).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.01.012.

References

[1] F. Keller, D.M. Pharr, Metabolism of carbohydrates in sinks and sources: galactosyl-sucrose oligosaccharides, in: E. Zamski, A.A. Schaffer (Eds.), Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships, Marcel Dekker, New York, 1996, pp. 157–183.
[2] R.L. Bieleski, Sugar alcohols, in: F.A. Loewus, W. Tanner (Eds.), Plant Carbohydrates. I. Intracellular Carbohydrates, Encyclopedia of Plant Physiology, vol. 13A, New Series, Springer Verlag, New York, 1982, pp. 158–192.
[3] M.H. Zimmermann, H. Ziegler, List of sugars and sugar alcohols in sieve-tube exudates, in: M.H. Zimmermann, J.A. Milburn (Eds.), Encyclopedia of Plant Physiology, Springer Verlag, Berlin, 1975, pp. 480–503.
[4] M. O'Neill, T. Ishii, P. Albersheim, A.G. Darvill, I.I. Rhamnogalacturonan, Structure and function of a borate crosslinked cell wall pectic polysaccharide, Annu. Rev. Plant. Biol. 55 (2004) 109–139.
[5] P.H. Brown, H. Hu, Phloem mobility of boron is species dependent: evidence for phloem mobility in sorbitol-rich species, Ann. Bot. 77 (1996) 497–506.
[6] S.G. Penn, H. Hu, P.H. Brown, C.B. Lebrilla, Direct analysis of sugar alcohol borate complexes in plant extracts by matrix-assisted laser desorption/ionization Fourier transform mass spectrometry, Anal. Chem. 69 (1997) 2471–2477.
[7] J.M.H. Stoop, J.D. Williamson, D.M. Pharr, Mannitol metabolism in plants: a method for coping with stress, Trends Plant Sci. 1 (1996) 139–144.
[8] T.H.H. Chen, N. Murata, Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes, Curr. Opin. Plant Biol. 5 (2002) 250–257.
[9] Z.F. Gao, L. Maurousset, R. Lemoine, S.D. Yoo, S. van Nocker, W. Loescher, Cloning expression, and characterization of sorbitol transporters from developing sour cherry fruit and leaf sink tissues, Plant Physiol. 131 (2003) 1566–1575.
[10] S. Yamaki, Properties and functions of sorbitol-6-phosphate dehydrogenase sorbitol dehydrogenase, sorbitol oxidase in fruit and cotyledon of apple (*Malus domestica* Mill. var. domestica Schneid), J. Jpn. Soc. Hortic. Sci. 49 (1980) 429–434.
[11] R. Lo Bianco, M. Rieger, S.J. Sung, Carbohydrate metabolism of vegetative and reproductive sinks in the late maturing peach cultivar 'Encore', Tree Physiol. 19 (1999) 103–109.
[12] K. Yamada, N. Niwa, K. Shiratake, S. Yamaki, cDNA cloning of NAD-dependent sorbitol dehydrogenase from peach fruit and its expression during fruit development, J. Hort. Sci. Biotechnol. 76 (2001) 581–587.
[13] Y. Oura, K. Yamada, K. Shiratake, S. Yamaki, Purification and characterization of a NAD(+)-dependent sorbitol dehydrogenase from Japanese pear fruit, Phytochemistry 54 (2000) 567–572.

[14] N.A. Bantog, K. Yamada, N. Niwa, K. Shiratake, S. Yamaki, Gene expression of NAD(+)-dependent sorbitol dehydrogenase and NADP(+)-dependent sorbitol-6-phosphate dehydrogenase during development of loquat (*Eriobotrya japonica* Lindl.) fruit, J. Jpn. Soc. Hortic. Sci. 69 (2000) 231–236.
[15] K. Yamada, Y. Oura, H. Mori, S. Yamaki, Cloning of NAD-dependent sorbitol dehydrogenase from apple fruit and gene expression, Plant Cell Physiol. 39 (1998) 1375–1379.
[16] S.W. Park, K.J. Song, M.Y. Kim, J.H. Hwang, Y.U. Shin, W.C. Kim, W.I. Chung, Molecular cloning and characterization of four cDNAs encoding the isoforms of NAD-dependent sorbitol dehydrogenase from the Fuji apple, Plant Sci. 162 (2002) 513–519.
[17] M. Nosarszewski, A.M. Clements, A.B. Downie, D.D. Archbold, Sorbitol dehydrogenase expression and activity during apple fruit set and early development, Physiol. Plant. 121 (2004) 391–398.
[18] X.L. Wang, Y.H. Xu, C.C. Peng, R.C. Fan, X.Q. Gao, Ubiquitous distribution and different subcellular localization of sorbitol dehydrogenase in fruit and leaf of apple, J. Exp. Bot. 60 (2009) 1025–1034.
[19] T.M. Kuo, D.C. Doehlert, C.G. Crawford, Sugar metabolism in germinating soybean seeds. Evidence for the sorbitol pathway in soybean axes, Plant Physiol. 93 (1990) 1514–1520.
[20] D.C. Doehlert, Ketose reductase activity in developing maize endosperm, Plant Physiol. 84 (1987) 830–834.
[21] K. Ohta, R. Moriguchi, K. Kanahama, S. Yamaki, Y. Kanayama, Molecular evidence of sorbitol dehydrogenase in tomato, a non-Rosaceae plant, Phytochemistry 66 (2005) 2822–2828.
[22] J. Jeffery, H. Jornvall, Enzyme relationships in a sorbitol pathway that bypasses glycolysis and pentose phosphates in glucose metabolism, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 901–905.
[23] K. Johansson, M. El-Ahmad, C. Kaiser, H. Jornvall, H. Eklund, J. Hoog, S. Ramaswamy, Crystal structure of sorbitol dehydrogenase, Chem. Biol. Interact. 130–132 (2001) 351–358.
[24] T. Pauly, J. Ekstrom, D. Beebe, B. Chrunyk, D. Cunningham, M. Griffor, A. Kamath, S. Lee, R. Madura, D. Mcguire, X-ray crystallographic and kinetic studies of human sorbitol dehydrogenase, Structure 11 (2003) 1071–1085.
[25] W. Maret, D.S. Auld, Purification and characterization of human liver sorbitol dehydrogenase, Biochemistry 27 (1988) 1622–1629.
[26] A.V. Sarthy, C. Schopp, K.B. Idler, Cloning and sequence determination of the gene encoding sorbitol dehydrogenase from *Saccharomyces cerevisiae*, Gene 140 (1994) 121–126.
[27] E. Haritatos, R. Medville, R. Turgeon, Minor vein structure and sugar transport in *Arabidopsis thaliana*, Planta 211 (2000) 105–111.
[28] M. Tanaka, I. Wallace, J. Takano, D. Roberts, T. Fujiwara, NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in *Arabidopsis*, Plant Cell 20 (2008) 2860–2875.
[29] O. Fiehn, J. Kopka, R.N. Trethewey, L. Willmitzer, Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadruple mass spectrometry, Anal. Chem. 72 (2000) 3573–3580.
[30] F. Kaplan, J. Kopka, D.W. Haskell, W. Zhao, K.C. Schiller, N. Gatzke, D.Y. Sung, C.L. Guy, Exploring the temperature stress metabolome of *Arabidopsis*, Plant Physiol. 136 (2004) 4159–4168.
[31] L. Rizhsky, H. Liang, J. Shuman, V. Shulaev, S. Davletova, R. Mittler, When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress, Plant Physiol. 134 (2004) 1683–1696.
[32] B. Ebert, D. Zöller, A. Erban, I. Fehrle, J. Hartmann, A. Niehl, J. Kopka, J. Fisahn, Metabolic profiling of *Arabidopsis thaliana* epidermal cells, J. Exp. Bot. 61 (2010) 1321–1335.
[33] P. Bais, S.M. Moon, K. He, R. Leitao, K. Dreher, T. Walk, Y. Sucaet, L. Barkan, G. Wohlgemuth, M.R. Roth, E.S. Wurtele, P. Dixon, O. Fiehn, B.M. Lange, V. Shulaev, L.W. Sumner, R. Welti, B.J. Nikolau, S.Y. Rhee, J.A. Dickerson, PlantMetabolomics.org: a web portal for plant metabolomics experiments, Plant Physiol. 152 (2010) 1807–1816.
[34] M. Nosarzewski, A.B. Downie, B. Wu, D.D. Archbold, The role of sorbitol dehydrogenase in *Arabidopsis thaliana*, Funct. Plant Biol. 39 (2012) 462–470.
[35] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassay with tissue culture, Physiol. Plant. 15 (1962) 473–497.
[36] T. Nakagawa, T. Kurose, T. Hino, K. Tanaka, M. Kawamukai, Y. Niwa, K. Toyooka, K. Matsuoka, T. Jinbo, T. Kimura, Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation, J. Biosci. Bioeng. 104 (2007) 34–41.
[37] L. Norambuena, L. Marchant, P. Berninsone, C.B. Hirschberg, H. Silva, A. Orellana, Transport of UDP-galactose in plants. Identification and functional characterization of AtUTr1, an *Arabidopsis thaliana* UDP-galactose/UDP-glucose transporter, J. Biol. Chem. 277 (2002) 32923–32929.
[38] S.M. de Sousa, M.G. Paniago, P. Arruda, J.A. Yunes, Sugar levels modulate sorbitol dehydrogenase expression in maize, Plant Mol. Biol. 68 (2008) 203–213.
[39] C.R. Somerville, S.C. Somerville, W.L. Ogren, Isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*, Plant Sci. Lett. 21 (1981) 89–96.
[40] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[41] L. Meng, L. Feldman, A rapid TRIzol-based two-step method for DNA-free RNA extraction from *Arabidopsis* siliques and dry seeds, Biotechnol. J. 5 (2010) 183–186.
[42] T. Czechowski, M. Stitt, T. Altmann, M.K. Udvardi, W. Scheible, Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*, Plant Physiol. 139 (2005) 5–17.
[43] C.L. Andersen, J.L. Jensen, T.F. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res. 64 (2004) 5245–5250.
[44] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[45] M. Handford, C. Rodríguez-Furlán, L. Marchant, M. Segura, D. Gómez, E. Alvarez-Buylla, G.Y. Xiong, M. Pauly, A. Orellana, Arabidopsis thaliana AtUTr7 encodes a Golgi-localized UDP-glucose/UDP-galactose transporter that affects lateral root emergence, Mol. Plant 5 (2012) 1263–1280.
[46] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[47] R. Jefferson, T. Kavanagh, M. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.
[48] J.M. Alonso, et al., Genome-wide insertional mutagenesis of *Arabidopsis thaliana*, Science 301 (2003) 653–657.
[49] R. Alcázar, J. Planas, T. Saxena, X. Zarza, C. Bortolotti, J. Cuevas, M. Bitrián, A.F. Tiburcio, T. Altabella, Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-expressing the homologous *Arginine decarboxylase 2* gene, Plant Physiol. Biochem. 48 (2010) 547–552.
[50] M. Klimacek, H. Hellmer, B. Nidetzky, Catalytic mechanism of Zn^{2+}-dependent polyol dehydrogenases: kinetic comparison of sheep liver sorbitol dehydrogenase with wild-type and Glu154 → Cys forms of yeast xylitol dehydrogenase, Biochem. J. 404 (2007) 421–429.
[51] J.L. Heazlewood, R.E. Verboom, J. Tonti-Filippini, I. Small, A.H. Millar, SUBA: the *Arabidopsis* subcellular database, Nucleic Acids Res. 35 (2007) D213–D218.
[52] M.R. Hanson, R.H. Köhler, GFP imaging: methodology and application to investigate cellular compartmentation in plants, J. Exp. Bot. 52 (2001) 529–539.
[53] R.J. Laby, S.M. Kincaid, D. Kim, S.I. Gibson, The *Arabidopsis* sugar-insensitive mutants sis4 and sis5 are defective in abscisic acid synthesis and response, Plant J. 23 (2000) 587–596.
[54] P. Richard, M.H. Toivari, M. Penttilä, Evidence that the gene YLR070c of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase, FEBS Lett. 457 (1999) 135–138.
[55] Y.S. Klepek, M. Volke, K.R. Konrad, K. Wippel, S. Hoth, R. Hedrich, N. Sauer, *Arabidopsis thaliana* POLYOL/MONOSACCHARIDE TRANSPORTERS 1 and 2: fructose and xylitol/H+ symporters in pollen and young xylem cells, J. Exp. Bot. 61 (2010) 537–550.
[56] F.B. Negm, W.H. Loescher, Detection and characterization of sorbitol dehydrogenase from apple callus tissue, Plant Physiol. 64 (1979) 69–73.
[57] H. Yamaguchi, Y. Kanayama, S. Yamaki, Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit, Plant Cell Physiol. 35 (1994) 887–892.
[58] Z.X. Guo, T.F. Pan, K.T. Li, F.L. Zhong, L. Lin, D.M. Pan, L.X. Lu, Cloning of NAD-SDH cDNA from plum fruit and its expression and characterization, Plant Physiol. Biochem. 57 (2012) 175–180.
[59] A. Cornish-Bowden, Fundamentals of enzyme kinetics, Portland Press, London, 1995.
[60] R. Velasco, et al., The genome of the domesticated apple (*Malus* × *domestica* Borkh.), Nat. Genet. 42 (2010) 833–839.
[61] J. Ito, T.S. Batth, C.J. Petzold, A.M. Redding-Johanson, A. Mukhopadhyay, R. Verboom, E.H. Meyer, A.H. Millar, J.L. Heazlewood, Analysis of the *Arabidopsis* cytosolic proteome highlights subcellular partitioning of central plant metabolism, J. Proteome Res. 10 (2011) 1571–1582.
[62] F.B. Negm, W.H. Loescher, Characterization and partial purification of aldose-6-phosphate reductase (alditol-6-phosphate: NADP 1-oxidoreductase) from apple leaves, Plant Physiol. 67 (1981) 139–142.
[63] D. Bartels, K. Engelhardt, R. Roncarati, K. Schneider, An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein, EMBO J. 10 (1991) 1037–1043.
[64] S. Yamaki, Subcellular localization of sorbitol-6-phosphate dehydrogenase in protoplast from apple cotyledons, Plant Cell Physiol. 22 (1981) 359–367.
[65] D. Winter, B. Vinegar, H. Nahal, R. Ammar, G.V. Wilson, N.J. Provart, An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets, PLoS ONE 2 (2007) e718.
[66] S.G. Mansfield, L.G. Briarty, The dynamics of seedling and cotyledon cell development in *Arabidopsis thaliana* during reserve mobilization, Int. J. Plant Sci. 157 (1996) 280–295.
[67] R. Tao, S.L. Uratsu, A.M. Dandekar, Sorbitol synthesis in transgenic tobacco with apple cDNA encoding NADPdependent sorbitol-6-phosphate dehydrogenase, Plant Cell Physiol. 36 (1995) 525–532.
[68] E.V. Sheveleva, S. Marquez, W. Chmara, A. Zegeer, R.G. Jensen, H.J. Bohnert, Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco. High amounts of sorbitol leads to necrotic lesions, Plant Physiol. 117 (1998) 831–839.
[69] M. Gao, R. Tao, K. Miura, A.M. Dandekar, A. Sgiura, Transformation of Japanese persimmon (*Diospyros kaki* Thunb.) with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase, Plant Sci. 160 (2001) 837–845.

[70] M. Deguchi, Y. Koshita, M. Gao, R. Tao, T. Tetsumura, S. Yamaki, Y. Kanayama, Engineered sorbitol accumulation induces dwarfism in Japanese persimmon, J. Plant Physiol. 161 (2004) 1177–1184.
[71] Y.S. Klepek, D. Geiger, R. Stadler, F. Klebl, L. Landouar-Arsivaud, R. Lemoine, R. Hedrich, N. Sauer, Arabidopsis POLYOL TRANSPORTER5, a new member of the monosaccharide transporter-like superfamily, mediates H(+)-symport of numerous substrates, including myo-inositol, glycerol, and ribose, Plant Cell 17 (2005) 204–218.
[72] A. Reinders, J.A. Panshyshyn, J.M. Ward, Analysis of transport activity of *Arabidopsis* sugar alcohol permease homolog AtPLT5, J. Biol. Chem. 280 (2005) 1594–1602.
[73] T.P. Michael, T.C. Mockler, G. Breton, C. McEntee, A. Byer, J.D. Trout, S.P. Hazen, R. Shen, H.D. Priest, C.M. Sullivan, S.A. Givan, M. Yanovsky, F. Hong, S.A. Kay, J. Chory, Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules, PLoS Genet. 4 (2008) e14.