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Effects of the Silencing of Sorbitol Dehydrogenase on Sugar Partitioning in Vegetative Sinks in Apple

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Summary

The major aim of this study was to determine the effect of the modulation of sorbitol dehydrogenase activity on sugar partitioning in apple leaves at different developmental stages. Transgenic plants with regulated sorbitol dehydrogenase (SDH) activity were obtained using transgenic sense and antisense technology. Plants were analyzed using native polyacrylamide gel electrophoresis assay and western blotting. Sugar levels (glucose, fructose, sorbitol and sucrose) were determined in leaves at different developmental stages. The two antisense lines (A090802-1 and A011803/A) showed reduced SDH activity in immature leaves while the sense lines (S071502 and S091202) showed similar SDH activity to untransformed controls. Western blot analysis partially correlated with activity assays implying the possibility of post-transcriptional regulation. Differences in SDH activity were associated with an altered fructose:sorbitol ratio in immature leaves of transgenic plants. Increased levels of glucose in immature and middle stage leaves were observed in antisense line A011803/A. Differences in fructose:sorbitol and sorbitol:sucrose ratios among transformants were not significant in mature, fully expanded leaves. These results suggest that the differential regulation of SDH activity in immature leaves could have dramatic consequences on the sugar partitioning between sink and source vegetative tissues and consequently on the vegetative growth of apple plants.

Key words. carbohydrate – leaves – *Malus* – SDH – sorbitol – transgenics

Introduction

Sugar alcohols, also known as polyols or polyalcohols, are the hydrogenated forms of aldoses or ketoses. Sorbitol is a major end-product of photosynthesis in many economically important fruit trees belonging to the *Rosaceae* family such as cherry, apple, pear, peach, apricot, and plum (Loescher et al. 1982). Many functions have been hypothesized for sorbitol, including translocation and storage of carbon, cryoprotection, prevention of activated oxygen species, boron transport, and energy delivery (Brown et al. 1999; Gao et al. 2001).

Sorbitol is synthesized in mature leaves by NADPH-dependent aldose-6-P reductase (S6PDH) that is essential for loading of photosynthates in plants that translocate sorbitol from leaves to sink tissues (Loescher et al. 1982). In sink tissue, sorbitol use is mediated by a NAD-dependent sorbitol dehydrogenase (SDH), which oxidizes sorbitol to fructose (Yamaguchi et al. 1994). S6PDH activity increases as SDH activity decreases during the conversion of apple leaves from sink to source (Loescher et al. 1982). In fruit, sucrose translocation stimulates starch synthesis while sorbitol triggers fructose synthesis. Plant SDH was first purified in 1994 (Yamaguchi et al. 1994) and the gene was cloned in 1998 (Yamada et al. 1998). Seasonal changes in the amount of NAD-dependent sorbitol dehydrogenase (SDH) protein were investigated in developing apple fruit (Yamaguchi and Kanayama 1996). The amount was very low in young fruits and rose during ripening. A weak correlation between enzyme protein and SDH activity suggests post-translational modification of pre-existing enzymes or isoenzymes of SDH.

In *Rosaceae* species, an increase in soluble carbohydrates during leaf development is usually due to increased accumulation of sorbitol. This increase is due to increased S6PDH and decreased SDH (Merlo and Passera 1991). Similar results were obtained in apple leaves (Loescher et al. 1982) and in apricot, where it was demonstrated that immature leaves cannot synthesize sorbitol (Bieleski and Redgwell 1985). Therefore, the inability to form polyols during the early stages of leaf development seems to be widespread in woody *Rosaceace*. The detection and quantification of SDH activity could be used to measure sink strength in plants that use sorbitol as the main translocated carbohydrate. Lo Bianco et al. (1999) suggested that sorbitol and sucrose play different roles in peach sinks, depending on the developmental stage of the sink (i.e., young versus mature fruit) and on the type of sink (i.e., reproductive versus vegetative organ). Sorbitol seems to have a predominant role in vegetative growth, where the activity of SDH correlates with shoot growth rate. Transgenic apple with reduced S6PDH activity accumulated sucrose and starch (Cheng et al. 2005) and bore fruits with more glucose and less fructose, starch, and malic acid without any differences in weight and firmness (Teo et al. 2006). In contrast, increased S6PDH activity led to sucrose accumulation. Interestingly, SDH activity and SDH transcription were specifically stimulated by exo-

genous sorbitol fed to shoot tips via the transpiration stomata but were specifically inhibited by glucose (Zhou et al. 2006). When sorbitol synthesis was decreased by antisense inhibition of S6PDH expression, cytosolic fructose-1,6-bisphosphatase (FBPase) activity *in vivo* was down-regulated to reduce the carbon flux to the cytosol and starch synthesis was consequently up-regulated in the chloroplast (Zrenner et al. 1996). These data showed that the photosynthetic system in sorbitol-synthesizing species has considerable plasticity. While sorbitol synthesis regulation in mature leaves (S6PDH regulation) is well studied, little is known about how SDH use is mediated by SDH in sink tissues.

The objective of this work was to obtain transgenic apple plants with an altered SDH activity and determine the effect of this modification on partitioning of different sugars during leaf development.

Materials and Methods

Plant transformation and selection of transgenic apple plants

Vectors containing SDH cDNA from apple in a sense and antisense orientation were constructed. The SDH gene was a RT-PCR product from *Malus domestica* cv. 'Greensleeves' RNA. RNA was isolated from young, newly expanded field grown leaves using Qiagen (Valencia, CA) RNEASY Plant mini kit. RT PCR was performed according to Promega's (Madison, WI) ACCESS RT-PCR kit. Primers were based on mRNA sequence (AB016256) of *Malus* × *domestica* Borkh.). The sequence for the forward primer was 5'-CAGCCTGAGAAAACAAAGAGAGAAAAATGGGAAAGGGAGGCATGTCTG-3'. The reverse primer sequence was 5'-TTACAGGTTAAACATGACCTTAATGGCATTGCC-3'. The cDNA product was cloned into Invitrogen's (Carlsbad, CA) PCR4-TOPO vector. DNA was isolated, sequenced by Davis Sequencing (Davis, CA) and compared to the AB016256 sequence. There were five base pair changes resulting in two amino acid changes (AA 59, arginine to lysine, and AA 104, histidine to aspartic acid). The same previously described primers were used to detect the presence of the SDH transgenic sequences in the regenerated lines using PCR analysis. The TOPO cloned SDH cDNA was ligated into a plant expression vector containing the 35S cauliflower mosaic virus promoter and the octopine synthase terminator in a sense and antisense orientation using the restriction enzyme EcoRI. This cassette was then ligated into the binary vector pDU99.2215 and introduced in *Agrobacterium tumefaciens* strain EHA101 as described by Tao et al. (1995). Apple transformation was similar to James et al. (1989) and involved *Agrobacterium*-mediated transformation of leaf discs from *Malus* × *domestica* (Borkh.) 'Greensleeves' (Teo et al. 2006). Selection was achieved with the antibiotic kanamycin and β-glucuronidase (GUS) activity in shoots was measured using fluorimetry (Jefferson et al. 1987). Plants were selected, GUS-positive shoots were rooted in the presence of kanamycin (James et al. 1989) and tested with PCR analysis for both the presence of nptII and SDH transgene. Two antisense lines (A090802-1 and A011803/A) and two sense lines, S071502 and S091202, were obtained. Plants were cultured *in vitro* for 6 months and then acclimatized in the greenhouse (average temperatures of 29 °C (highest) and 14 °C (lowest) and allowed to grow on their roots in pots for one vegetative season. The plants were irrigated with an automatic system each morning, fertilized once a week. Plants were not subjected to artificial light (16 h of photoperiod, 1000 µMol of light on weekly average) except from September to November. Fertilizer was composed by calcium nitrate concentrated solution (0.5 M) injected at a rate of 1:120 or 0.8 %) and GrowMore 4-18-38 with micronutrients, plus magnesium sulfate concentrated solution (1.2 M) injected at a rate of 1:200 or 0.5 %).

Transgene copy number quantification

Approximately 100 mg of leaf tissue from all transgenic plants was ground to a fine powder in liquid nitrogen. DNA was isolated by using a DNeasy Plant Mini kit (Qiagen Inc., USA) according to the manufacturer's instructions. Each DNA sample concentration was adjusted to 10 ng μL^{-1} and used for real-time PCR analysis. Plasmid DNA (pDU02.0908) was isolated by using a QiaPrep Miniprep kit (Qiagen Inc., USA) according to the manufacturer's instructions. The DNA concentration was measured by using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Primers and probes were designed to amplify the SDH transgene by using Primer Express software (Applied Biosystem Co., USA), following the manufacturer's instructions. Sequences of primers were the following: GGAGGAAGGCGCGATGT (Forward) and CCGGCGACAAGCATGAAT (Reverse). Probe sequence for Taqman Real Time PCR analysis was the following: TGAGCCCTTAAGTGTTGG. Optimum primer and probe pairs, as predicted by the software, were used. TaqMan Universal PCR Master Mix was supplied by Applied Biosystem Company for TaqMan reactions. The amplification reaction was performed in a total reaction volume of 12 µl. Reactions included 5 µl of template DNA, 6 µl of TaqMan Universal PCR Master Mix (2X), 0.58 µl Small Scale Assay Mix (which includes forward primer, reverse primer and probe) and sterile molecular biology grade water to a total volume of 12 µl. All PCR reactions were performed in 48-well plates and the exact reaction cycling conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification and post-run data analysis were carried out on an ABI Step One v2.0 system (Applied Biosystems, USA). All primer and probe concentrations were optimized. Negative controls containing water as template was subjected to the same procedure. Each sample was run in triplicate. The Ct value was determined by using the instrument's software. A serial dilution of linearized plasmid DNA was used to generate a standard curve. The number of g $molecule^{-1}$ also known as copy number was calculated as follows:

Weight in Daltons (g mol^{-1}) = (bp size of plasmid + insert) (330 da × 2 nucleotides bp^{-1})

Number of molecule was determined as follows:

Concentration of plasmid (g μl^{-1})/copy number = $(178 \times 10^{-9}$ g $\mu l^{-1})/(2.27 \times 10^{-17}$ g $molecules^{-1})$ = 0.78×10^{6} molecules μl^{-1}.

Ten serial dilutions were performed (dilution factor: 1:10) and standard curve was constructed.

Protein extraction

Immature leaves (1–2 cm) were collected for protein extraction in spring (March–April) and immediately put on ice. Leaves (0.25–0.5 g) were ground with mortar and pestle pre-chilled at 4 °C in 1–2 ml of buffer D (8 mM citric acid, 5.7 mM cysteine HCl monohydrate, 1.25 mM polyethylene glycol 8000, 0.125 g ascorbic acid, 0.813 g Trizma base, 27.5 ml glycerol, and dH_2O up to 125 ml; ratio 1 mg tissue: 4 ml buffer volume) containing 50–100 mg insoluble polyvinylpolypyrrolidone (PVPP). During grinding, 1–2 µl Protease Inhibitor Mix (GE Healthcare Life Sciences) and 50–100 mg PVPP were added to the sample and the material was centrifuged 20 min at 20000 g at 4 °C. Sufficient supernatant to contain 300 µg protein, determined using the Bradford assay (Bradford 1976) was used for gel analysis.

Native PAGE electrophoresis and assays. Native PAGE electrophoresis was performed using a separating gel preceded by a stacking gel. The separating gel was composed of 3.4 ml 1.5 M Tris-HCl, pH 8.8, 4.4 ml 30% acrylamide, 5.6 ml dH_2O, 67 µl ammonium persulfate (APS, 10% w/v), and 15 µl TEMED. The stacking gel was composed of 0.9 ml 0.5 M Tris-HCl, pH 6.8, 0.6 ml 30 % acrylamide, 2.2 ml dH_2O, 11.25 µl APS (10 % w/v), and 3 µl TEMED. The two solutions were degassed for 5 min before pouring and allowed to polymerize for 30 min each. 300 µg protein sample was loaded per lane. Loading dye contained 2 % SDS w/v and added to the sample before boiling. Samples were boiled for 10 min in the presence of the loading dye prior to loading them on the gel. Gel electrophoresis was performed for one hour at 100–110 V at 4 °C in running buffer (3 g L^{-1} Trizma base and 7.2 g L^{-1} glycine in dH_2O). Gels were incubated at 37 °C overnight in 50 ml reaction solution (10 ml 0.5 M glycine, pH 9.5, 7.5 mg 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), 2.5 mg Phenazine methosulfate (PMS), 15 mg NAD, 250 mg sorbitol, and dH_2O up to 50 ml). Statistical analysis was performed by measuring the intensity (in arbitrary units) of the gel band with AlphaImager (AlphaInnotech). Three different replicates using protein extracts from three different plants were used. ANOVA univariate analysis was performed using the Duncan test (P = 0.05).

WESTERN blot analysis. Gel preparation and electrophoresis were performed as described for native PAGE except that SDS was included in the gel and running solutions at the final concentration of 0.1 %. 100 µg protein (as determined by Bradford assay) were used per sample. After electrophoresis, the protein was transferred from the gel to a nitrocellulose membrane using a Trans-blot apparatus (Invitrogen; Carlsbad, CA). Trans-blotting buffer (TBST) was composed of 10 mM Tris, 150 mM NaCl, and 0.05 % Tween 20. The membrane was immersed in a container with blocking solution (5 % nonfat milk in TBST) and incubated with gentle shaking at room temperature for 30 min. Blocking solution was decanted and replaced with a solution of 0.5 % BSA-TBST containing the primary antibody specific for apple SDH at 1:1000 and incubated for 30 min at room temperature with gentle shaking. The SDH antibody was made against commercially obtained sheep liver SDH (Sigma, St. Louis, MO) in rabbit (Fujii 1992). After the removal of the final wash, the secondary antibody was added (4.8 µl Anti-Rabbit IgG) to 20 ml of 0.5 % BSA-TBST and incubated for 30 min. The secondary antibody solution was then removed, and washing was performed three times for 5 min each with TBST buffer. Chemiluminiscent method was used for the detection of the reaction between antibody and SDH protein. The membrane was photographed with a Kodak film using the instructions of PerkinElmer (Waltham, MA, USA).

Carbohydrate analysis

Immature leaves, intermediate leaves (light green, completely open, not fully expanded but photosynthesizing), and fully expanded mature leaves were harvested from transgenic and control 'Greensleeves' apple trees and immediately put on ice until analysis. Three to five biological replicates were used for each leaf stage for each transgenic or control line. 100 mg of each sample was homogenized 1 min at 30 Hz^{-1} in 1 ml ethanol and Tris-HCl 0.1 M solution (pH > 7) with 10 mg of inositol as an internal standard (Teo et al. 2006). Samples were kept on ice for 30 min and centrifuged (4000 g) 5–10 min at room temperature, after which supernatant was recovered. These actions were repeated 4 times to collect 4 ml per sample. Samples were dried under nitrogen in a water bath at 40–50 °C until a small quantity of gelatinous material remained. This was resuspended in 1 ml 0.01 M Tris-HCl buffer and loaded onto Sep-pack cartridges (Perez et al. 1997). Soluble sugars were eluted with 4 ml water and filtered. An aliquot (1 ml) and sugar standards (glucose, fructose, sucrose, sorbitol) were subjected to HPLC-MS analysis (Perkin Elmer, CA, USA). Results were expressed as the peak height (mm) and the concentration of each sugar (mg g^{-1} fresh tissue) was determined using the following formula:

$$\text{Sugar concentration} = (10*\text{Height}_{sugar}*\text{Standard}_{inositol})/(\text{Standard}_{sugar}*\text{Height}_{inositol}*\text{Fresh tissue (g)})$$

Data were analyzed using Constat statistical software to perform ANOVA univariate analysis and a "post hoc" Duncan test.

Results

We obtained two antisense transgenic lines, A090802-1 and A011803/A showing an abnormally short growth and precocious loss of leaves during the spring. In addition, line A090802-1 showed clear stress-related symptoms such as curled and bleached leaves, reduced leaf size and tree height, premature leaf loss, and increased growth of axillary shoots close to the apex (Fig. 1).

Transgenic sense lines S071502 and S091202 were also obtained and no phenotypic differences were observed in comparison to wild-type (Fig. 2).

Transgene copy number determination of transgenic plants was performed using Real Time PCR and genomic DNA (Table 1).

Based up on the standard curve generated by using the plasmid DNA, in 50 ng of plant genomic DNA, the sense plant S091202 contains a single SDH transgene copy number. The other sense plant, S071502 contained two copies of SDH transgene while anti-sense plants A090802-1 and A011803/A possessed 6 and 4 copies of the transgene, respectively.

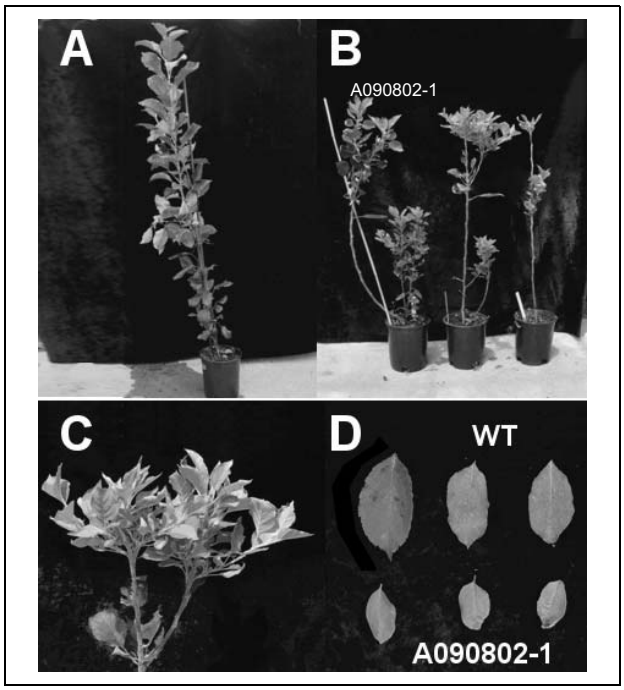


Fig. 1. Habitus of 'Greensleeves' wild-type plants (A) and antisense line A090802-1 (B). Transgenic plants showed precocious loss of leaves during spring, short growth, and loss of apical dominance. Close-ups of the apex for A090802-1 (C) and leaf comparison (D).

Table 1. Transgene quantification using Real Time PCR. Ct values, copy 5µl^{-1} and copy g plant^{-1} were indicated for all transgenic lines.

Transgenic lines	Ct Value	copy 5µl^{-1}	Copy g plant^{-1}
S071502	15.3	2.36E+06	9.45E+07
S091202	35.5	1.25E+00	5.01E+01
A090802-1	20.3	6.75E+04	2.70E+06
A011803/A	30.4	4.98E+01	1.99E+03

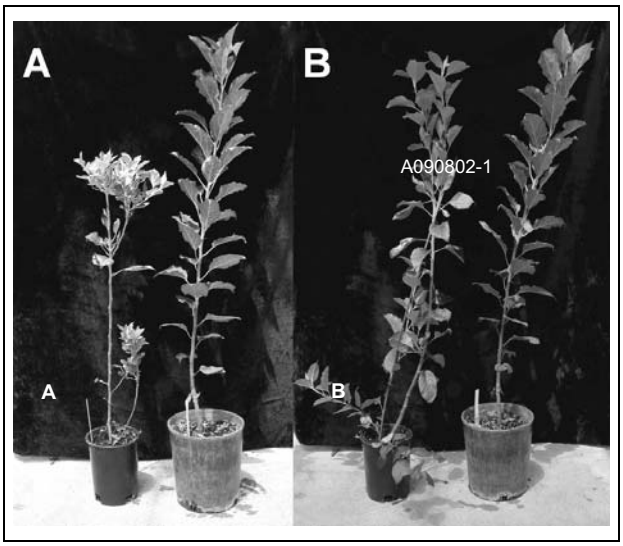


Fig. 2. A) Habitus of 'Greensleeves' transgenic antisense line A090802-1 (left) and wild-type plants (right). B) "Greensleaves" trangenic sense line S071502 (left) and wild-type (right) plants.

Table 2. SDH (sorbitol dehydrogenase) activity in immature leaves of transgenic and control 'Greensleeves' apple trees. Activity is reported in arbitrary units of intensity from protein samples separated by native polyacrylamide gel.

'Greensleeves' lines	Signal intensity (Arbitrary Unit)
Control (wild-type)	81.0 (4.3) b
A090802-1	55.5 (3.3) a
A011803/A	63.2 (2.7) a
S071502	73.0 (5.6) b
S091202	88.0 (3.9) b

Values are means of three different replicates (standard deviation). Different letters indicate significant differences by ANOVA univariate analysis using the Duncan test as a post hoc test (P = 0.05)

At the beginning of the vegetative growing season (March), SDH activity was measured in immature leaves from transgenic and wild-type lines using gel-activity electrophoresis. Antisense lines A090802-1 and A011803/A showed significantly less activity than the wild-type (Table 2, Fig. 3).

On the contrary, the sense lines S071502 and S091202 showed no significant differences from wild-type leaves. This evidence was consistent with the phenotypic observation of transgenic lines. Reduced protein activity might occur in transgenic lines where sufficient antisense RNA is produced to hybridize with the sense RNA transcribed from the endogenous SDH genes. The similar SDH activities between the transgenic sense lines and the control are consistent with the lack of phenotypic differences between these lines.

To complement the enzyme activity data, we used Western blotting analysis. A fragment of ~ 43 kDa (between 37 and 50 kDa molecular markers) was detected in transgenic lines S071501 and A090802-1 and in wild-type leaves (Fig. 4).

This band corresponds to the reported size of 42 kDa (Yamada et al. 1998). Line S071502 showed the most intense band while antisense line A090802-1 had a less intense band than the wild-type. No analysis of line A011803/A and S091202 was possible for insufficient plant material.

Concentrations of the main carbohydrates were determined in immature, intermediate (not fully expanded) and mature leaves in transgenic and wild-type lines. In immature leaves, lines A090802-1 and A011803/A had a lower fructose:sorbitol ratio than did control (Table 3).

Altered sorbitol:sucrose ratios were also observed in A011803/A (lower) and S071502 (higher) compared to controls. Considering sugars separately, line A011803/A had more glucose and less fructose than controls.

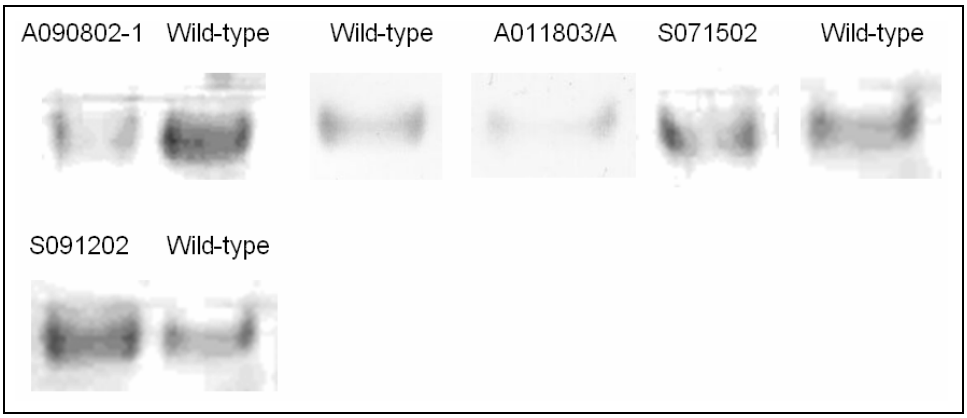


Fig. 3. Activity of SDH using native gel electrophoresis in transgenic and wild-type immature apple leaves. Statistical analysis was performed measuring intensity (arbitrary unit) of the gel band with Alphaimager software. Three different replicates using protein extracts from three different plants were used. ANOVA univariate analysis was performed using the Duncan test (P = 0.05).

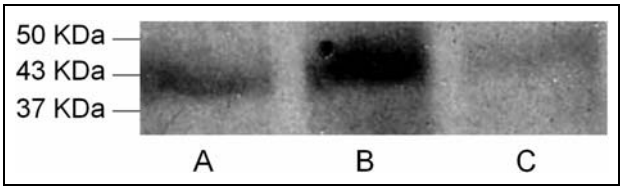


Fig. 4. Western blotting of protein extract from sense and antisense 'Greensleeves' lines and untransformed controls (wild-type): (A) wild-type, (B) S071502, (C) A090802-1. The molecular weights were indicated on the left.

Transgenic intermediate leaves showed no significant differences from the controls in the fructose:sorbitol ratio (Table 3), although there was a significant difference between lines A090802-1 and S071502. Antisense line A090802-1 had an increased sorbitol:sucrose ratio in comparison to antisense line A011803/A and the wild type, but there were no differences among the other lines. Significantly increased levels of glucose was also observed in intermediate leaves of line A011803/A as occurred in immature leaves.

Table 3. Soluble carbohydrate concentrations in immature, intermediate and mature leaves from transgenic and wild-type 'Greensleeves' lines.

Lines and Stage	Glucose (mg g^{-1})	Sucrose (mg g^{-1})	Fructose (mg g^{-1})	Sorbitol (mg g^{-1})	Fructose:sorbitol ratio	Sorbitol:sucrose ratio
Immature leaves						
Wild-type	5.18 (1.1) a	3.81 (3.2) a	17.38 (6.9) b	11.54 (4.0) a	1.51 (0.3) b	3.02 (0.8) b
A011803/A	16.99 (1.5) b	5.05 (1.3) a	6.93 (2.1) a	8.94 (2.2) a	0.77 (0.4) a	1.77 (0.4) a
A090802-1	4.93 (0.9) a	6.44 (0.2) b	18.10 (1.0) b	20.76 (3.5) b	0.87 (0.1) a	3.22 (0.8) b
S071502	3.38 (0.9) a	2.12 (1.9) a	21.57 (7.3) b	9.76 (2.4) a	2.21 (0.4) c	4.60 (0.7) c
S091202	3.50 (1.0) a	2.98 (0.3) a	27.04 (7.4) b	8.20 (2.4) a	3.29 (0.3) c	2.75 (1.0) b
ANOVA	*	*	*	*	*	*
Intermediate leaves						
Wild-type	4.50 (1.2) a	7.84 (4.9)	20.73 (9.4) ab	11.55 (4.0) ab	1.79 (0.9) ab	1.47 (0.3) a
A011803/A	13.35 (1.4) b	4.64 (3.6)	7.45 (4.2) a	9.49 (1.0) a	0.78 (0.1) ab	2.04 (0.5) a
A090802-1	2.79 (0.9) a	7.57 (0.9)	10.76 (4.3) a	21.24 (4.3) b	0.51 (0.2) a	2.81 (0.1) b
S071502	4.85 (2.6) a	6.36 (1.2)	37.40 (7.7) b	13.02 (3.9) ab	2.87 (0.3) b	2.05 (0.4) ab
S091202	5.20 (2.7) a	8.39 (1.2)	32.95(10.7) ab	14.55 (3.9) ab	2.26 (0.3) b	1.73 (0.4) ab
ANOVA	*	ns	*	*	*	*
Mature leaves						
Wild-type	4.63 (2.0)	10.39 (1.0)	31.54 (5.9)	16.35 (4.3)	1.93 (1.3)	1.57 (0.3)
A090802-1	6.78 (3.6)	12.83 (4.4)	33.00 (8.4)	18.27 (5.1)	1.81 (0.3)	1.42 (0.1)
S071502	2.97 (1.9)	8.20 (2.3)	39.79 (6.3)	11.94 (2.0)	3.33 (2.2)	1.45 (0.1)
S091202	4.03 (1.8)	7.59 (3.3)	28.15 (7.8)	9.86 (4.4)	2.85 (1.2)	1.29 (0.6)
ANOVA	ns	ns	ns	ns	ns	ns

* Different letters indicate significant differences by ANOVA univariate analysis using the Duncan test as a post hoc test (P = 0.05)

Discussion

Although SDH activity was detected previously in immature peach leaves (Merlo and Passera 1991), results presented here are the first to use transgenic plants with differentially regulated SDH expression and activity in vegetative sinks to determine the effects on phenotype and sugar partitioning. The transgenic antisense lines A090802-1 and A011803/A showed clear stress-related symptoms such as curled and bleached leaves, reduced leaf size and tree height, premature leaf loss, and increased growth of axillary shoots close to the apex. At the beginning of the growing season, the antisense lines showed significantly reduced SDH activity, while the sense line S071502 was similar to the control.

In 'Fuji' apple, a gene family encodes different SDH isoforms (Park et al. 2002). These proteins are divided into two groups based on amino acid identity. One group included MdSDH2 (Genbank accession AF323505), MdSDH3 (AF323506), and MdSDH4 (AF323507), which showed 90–92 % identity with each other. The other group was represented only by MdSDH1 (accession AF323504), which has only 69–71 % identity to the first group. Expression of MdSDH2, MdSDH3, and MdSDH4 were restricted to sink tissues such as young leaves, stems, roots, and maturing fruit, while MdSDH1 was expressed in both immature and mature leaves (Park et al. 2002). Our sequence has a closer similarity to MdSDH1 than to other sequences such as MdSDH2, MdSDH3, or MdSDH4. It is possible that the decrease of SDH activity observed in antisense lines (Table 2) was mostly due to the down-regulation of MdSDH1. In this case MdSDH1 is probably the most responsible for sorbitol transformation to fructose in vegetative sink tissues. Other reports are inconsistent in reporting the size of the apple SDH protein. Yamaguchi et al. (1994) reported a 62 kDa protein and another study reported one at 42 kDa protein (Yamada et al. 1998). Nosarszewski et al. (2004) observed a molecular mass of 37 kDa although the SDH sequence length suggested a protein between 39 and 40 kDa. These differences suggest a possible different initiation site for SDH translation or post-translational modification of SDH protein length. We found the lowest SDH concentration in A090802-1, consistent with the low SDH activity in this line (Table 2), while S071502 showed a more intense band than the control (Fig. 4). These data only partially agree with the determined SDH activity. However, post-translational modification of an inactive SDH pool that affects activity was previously suggested (Yamaguchi and Kanayama 1996). SDH activity could be regulated by activation or inactivation of the pre-existing enzyme. A second alternative is that different SDH isoforms are produced at different times and differ in their kinetics. This would be consistent with the discovery of a gene family for SDH (Park et al. 2002).

These differences were clearly linked to the abnormal phenotype of these trees and probably induced the precocious leaf loss. Most of the glucose existing in apple sink tissues is produced by conversion of sorbitol to glucose, catalyzed by sorbitol oxidase (SOX) (Zimmermann and Zeigler 1975). Sucrose can also be converted into fructose and glucose, but usually in a lower quantity than sorbitol (Daie 1993). It is possible that decreased SDH activity may influence the glucose concentration, making more sorbitol available for the reaction catalyzed by sorbitol oxidase. Also, sucrose is an alternative substrate to sorbitol for production of fructose and glucose through the activity of invertase. However, only line A090802-1 had an altered sucrose concentration (Table 3).

The fructose:sorbitol ratio can be a valid index of SDH activity. Immature leaves of sense line S071502 showed a significant increase in this ratio while antisense lines A011803/A and A090802-1-1 had a decrease, agreeing with the lower SDH activity previously observed.

Sorbitol:sucrose is another ratio usually used to determine the efficiency of sorbitol and sucrose metabolism. In immature leaves, this ratio was highest in S071502 and lowest in A011803/A. In intermediate leaves, at least some photosynthesis was taking place, but the leaves were in transition from sink to source. SDH might still have been active even if carbon partitioning was also affected by photosynthesis. Although there were no significant changes to the fructose:sorbitol ratio in the transgenic lines, SDH activity is hypothesized to influence sugar partitioning along with other enzymes such as S6PDH, SPS (sucrose phosphate synthase), and FBPase (fructose-1,6-bi-phosphatase).

As expected, in mature leaves no significant differences among these lines were detected in either sugar ratio, since transcription of the SDH gene is specifically induced in sink tissues. The A011803/A line was not analyzed at this stage because precocious leaf drop left no mature leaves available. As expected, more variability in sugar concentrations within lines was found in mature leaves (Table 3), where photosynthesis is active and several related enzymes (fructose-1,6-biphosphatase, S6PDH, and sucrose-phosphate-synthase) can produce high variability in sugar determination (Daie 1993).

In conclusion, different transgenic apple trees with altered SDH activity were obtained in this study. Vegetative disorders such as a shorter growth, precocious spring leaf loss, loss of apical dominance, and excessive growth of axillary shoots close to the apex were observed in transgenic lines with reduced SDH activity. These differences were linked to an altered fructose:sorbitol ratio in immature leaves. The sense line S071502 showed similar SDH activity and vegetative characteristics to the wild-type. These results suggest that the differential SDH activity in immature leaves could have dramatic consequences on the sugar partitioning and consequently on the vegetative growth of apple plants.

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